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**Towards the Development of
Protein-Protein Interaction
Standards to Improve the
Comparability of Protein
Interaction Measurements**

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ABSTRACT

In order to improve the inter-platform comparability of protein-protein interaction measurements, there is a need for appropriate protein-protein interaction model systems to be developed and assessed. This report summarises work carried out, under the Measurements for Biotechnology (MfB) Programme 2004-2007, towards the development and characterisation of such model systems.

By reviewing the needs for the measurement of protein-protein interactions, two model systems were selected, the *E. coli* multi-subunit enzyme Tryptophan Synthase, and the *E. coli* N Utilisation substance A / Phage Lambda N antitermination protein interaction pairs, were selected as suitable model systems. These were produced in-house, and assessed using two industrially important platforms: isothermal titration calorimetry (ITC) and surface plasmon resonance (SPR).

Published protein expression and purification protocols were modified to improve their robustness and the binding activity of each interaction pair was successfully characterised using the two orthogonal measurement platforms. The values for binding affinity, generated using the two techniques, agreed well. It was found, however, that in the case of SPR, this agreement was dependent on the surface immobilisation strategy used. Both interaction pairs were assessed as potential protein-protein interaction standards and it was concluded that only the TrpA/TrpB pair appeared suitable.

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Abbreviations

AUC	analytical ultracentrifugation
bp	base pair
DPI	dual polarisation interferometry
DTT	dithiothreitol
EDC	1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
Fc	flow-cell
FRET	fluorescence resonance energy transfer
IPTG	isopropyl β -D-1-thiogalactopyranoside
ITC	isothermal titration calorimetry
LamN	phage Lambda N antitermination protein
MALDI-TOF	matrix assisted laser desorption ionisation time-of-flight
MS	mass spectrometry
NHS	<i>N</i> -hydroxysuccinimide
NLLS	non-linear least squares
NSB	non-specific binding
NusA	N-utilisation substance A
P20	Polysorbate 20
PCR	polymerase chain reaction
PLP	pyridoxal-5'-phosphate
RU	response units
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SPR	surface plasmon resonance
Sulfo-MBS	<i>m</i> -maleimidobenzoyl- <i>N</i> -hydroxysuccinimide ester
TrpA	tryptophan synthase α -subunit
TrpB	tryptophan synthase β -subunit

1 Introduction

1.1 Context

This report has been produced as part of the work package entitled “To improve the comparability of protein interaction measurement parameters” contained within the project “More comparable measurement of higher order structure and interactions of proteins” under the Department of Trade and Industry’s second “Measurements for Biotechnology” programme. Further information about this project, and others under the programme, can be obtained at: <http://www.mfbprog.org.uk> or <http://www.npl.co.uk/biotech>.

1.2 Scope

The precise and accurate measurement of the interaction affinity between a protein and another biomolecule, be it a second protein, DNA target or small molecule drug, is vital to drug discovery, lead optimisation, quality control and many areas of medical diagnostics.

The technology platforms utilised within industry for these measurements, range from low throughput methods such as isothermal titration calorimetry (ITC) to higher throughput methods such as ELISA and protein microarrays. However, inter-platform comparability is often lacking for many of these techniques. There is a need to address this issue, and one approach is through the development of well characterised interaction pairs that can be applied across multiple platforms to ensure comparability.

The development of suitable interaction pairs would expedite the accurate discrimination of ‘real’ interactions from false positives and false negatives. This would greatly impact the lead identification stage of drug discovery for both the pharmaceutical and biopharmaceutical sectors, and in addition help validate and link diagnostic results obtained using point of care (POC) instruments with those obtained in the clinical labs.

To this end this report details the work carried out towards the development of such interaction pairs and assesses their application as ‘inter-platform comparability standards’.

For the purposes of this report the emphasis will be placed on protein-protein interactions. The following review papers provide further information on protein-DNA interactions [1-4], protein-lipid binding [5, 6] and protein-drug interactions [7-9].

1.3 Background

1.3.1 Protein-Protein Interactions

Protein-protein interactions are vital for the control of nearly every cellular process. For this reason most drugs, be they traditional small molecule drugs or larger biomolecule therapeutics, target these interactions in disease pathways. The identification and characterisation of the multitude of protein-protein interactions that

exist in the human proteome is now the next great hurdle to fully understanding the molecular processes that regulate our cells and tissues. The strengths of protein-protein interactions cover a wide range of affinities and this diversity makes it impossible to measure every affinity using only a single technique, as no one technique has a suitably broad dynamic range. In addition protein-protein complexes are not always simple 1:1 interactions and are often found to contain multi-subunits, for example pyruvate dehydrogenase, which contains 3 subunits [10] and the DNA replication complex of *E. coli* that consists of up to 18 subunits [11]. In fact protein-protein interactions can vary from homo-oligomerisation, as seen in the streptavidin tetramer, hetero-oligomerisation as seen in haemoglobin, to heterologous complexes observed from numerous families of signal proteins and immunoglobulins.

These factors, along side the diversity of the protein molecules themselves, combine to make detection, measurement and comparison of protein-protein interactions far from simple.

The biophysical properties of any given protein-protein interaction are used to define and categorise it. These properties are also very diverse and sometimes hard to elucidate, as there are a number of forces that govern the formation of a protein-protein complex. These forces include electrostatic forces, hydrogen bonds, van der Waals contacts and hydrophobic effects. The last of these forces, as with protein folding, is considered to be the primary driver behind protein-protein complex formation and determines the strength of the interaction, while the remaining forces govern the specificity.

1.4 Methods for the Detection and Analysis of Protein-Protein Interactions

Most methods can be crudely separated into two groups. Those that identify protein-protein interactions and those that provide information on the biophysical properties of a given interaction.

1.4.1 Identification of Protein-Protein Interactions

There are many different methods for the detection and identification of protein-protein interactions and only a select few will be discussed in this report [12]. These methods can be *in vivo* or *in vitro* based, and certain of the methods can employ ligand libraries.

Two classical methods used for the identification of protein-protein interactions are those of immunoprecipitation and affinity blotting. Affinity blotting employs polyacrylamide gel electrophoresis (PAGE) to separate proteins from one another after which they are transferred to a membrane much like in Western blotting. Instead of using an antibody, however, a labelled protein of interest is used to identify any binders within the population of proteins immobilised on the membrane [13].

Immunoprecipitation has been widely used to identify protein-protein complexes and involves the coimmunoprecipitation of a protein probe and its unknown binding partner, from a cell lysate, for example, with a specific antibody. This precipitate is then separated from the other proteins and washed prior to elution of the unknown interacting protein. An example of the application of this technique is in the identification of Rb protein as a binding partner for the adenovirus E1A protein [14].

Current methods now utilise Protein A or G linked beads for the precipitation of the antibody/antigen complexes.

While the techniques described above are not library-based, techniques that employ libraries are important. One common library-based, *in vivo* method used for the identification of protein-protein interactions is the two-hybrid system [12]. This is a genetic method that detects the interaction of two proteins using the transcriptional activity of a reporting gene such as luciferase [15, 16]. The technique relies on the fact that the DNA binding domain and transcriptional activation domain of certain site-specific transcription factors do not have to be covalently linked to remain functional and merely require that they be in close proximity to one another.

Phage display is another powerful library-based technique that enables thousands of protein variants to be screened simultaneously for binding activity to a chosen ligand. The technique involves expressing proteins as fusions to bacteriophage particles and then screening these displayed proteins against ligands of interest through rounds of affinity assays known as panning [17, 18]. The stringency of these panning steps can be varied and therefore used to select binders of varying strengths. This technology is now used very successfully within the biotechnology industry to produce human antibody-based therapeutic products. An example is HUMIRA® from Cambridge Antibody Technology (CAT), that targets Tumour Necrosis Factor Alpha (TNF α), a protein that is produced by the cells of the immune system in patients with rheumatoid arthritis.

1.4.2 Biophysical Analysis of Protein-Protein Interactions

Section 1.4.1 focussed on methods that detect and identify protein-protein interactions. This section will describe some of the most commonly used techniques for measuring the biophysical properties of protein-protein interactions. The more established methods include optical spectroscopy, isothermal titration calorimetry (ITC) as well as surface plasmon resonance (SPR) and analytical ultracentrifugation (AUC). Two emerging techniques will also be covered, dual polarisation interferometry (DPI) and mass spectrometry (MS).

1.4.2.1 Optical Spectroscopy

Fluorescence is the dominant optical method used for the study of protein-protein interactions. It is far more sensitive than other optical techniques because of the low background signal and the sensitivity of the fluorophore to environmental changes [19, 20].

By labelling one protein, the binding event can be followed using a technique known as fluorescence anisotropy, which measures the extent of the depolarisation of the light emitted by the fluorophore after excitation with polarised light [21]. The magnitude of depolarisation is proportional to the rate at which the fluorophore and hence the covalently linked protein is tumbling in solution. As the tumbling of molecules in solution is proportional to their size, any change due to the binding of another molecule affects the amount of depolarisation observed. This technique is widely used in high throughput drug discovery due to its speed and sensitivity [22, 23].

When both binding partners are labelled with fluorophores that have overlapping emission/excitation spectra it becomes possible to follow the binding event by

measuring a phenomenon known as fluorescence resonance energy transfer (FRET). This phenomenon only occurs when the two fluorophores are within 100 Å of each other and their transition dipoles are appropriately orientated [24]. The labelling of the proteins and the FRET measurements are usually carried out *in vitro*, however, the use of green fluorescent protein (GFP) as a fusion has allowed binding measurements to be carried out *in vivo* [25, 26]. Dissociation constants over the range 10^{-6} - 10^{-11} are easily measured.

1.4.2.2 Isothermal Titration Calorimetry (ITC)

ITC measures the heat changes that occur upon the interaction of two molecules in solution. Unlike most optical techniques (Section 1.4.2.1) ITC does not require the proteins to be labelled. Neither do they need to be surface immobilised as is the case for SPR (Section 1.4.2.4) and DPI (Section 1.4.2.5), thus the binding event is measured under true, in-solution conditions [27, 28]. ITC is classed as an information rich technique due to the fact that it provides a comprehensive determination of all of the thermodynamic changes (ΔH , ΔG and ΔS) for an interaction, in addition to the binding constant (K_A) and the stoichiometry of the interaction [29]. If the interaction is measured at different temperatures, the change in heat capacity (ΔC_p) can be calculated using a Van't Hoff plot. This provides further information regarding the hydrophobic nature of the interaction [29].

The changes in heat associated with protein-protein binding events are generally small, and this means that the sensitivity of this technique is low compared to that of fluorescence or SPR. For this reason relatively large quantities of material are required for each measurement and this can place restrictions on its use. In addition, measurements are traditionally slow, which precludes the use of ITC as a high throughput technology. Recent advances in instrumentation, however, now allow single injection methods to be used for yes/no screening measurements at far higher throughputs than before [30, 31]. Protein-protein interactions with K_D over the range of 10^{-3} - 10^{-9} can be measured normally. If a competition assay is designed then ITC can be used to measure K_D down to 10^{-11} [32].

1.4.2.3 Analytical Ultracentrifugation (AUC)

AUC is a technique which is returning to favour as a tool for analysing protein-protein interactions. The technique involves measuring the sedimentation of interacting macromolecules in a centrifugal field in the absence of any labelling, and without the contribution of a surface. These measurements allow the hydrodynamic and thermodynamic properties of the associating macromolecules to be calculated [33, 34]. Two techniques can be used to measure binding events depending on whether the interaction is static (slowly reversible) or dynamic (rapidly reversible) relative to the timescale of the experiment. For static associations sedimentation velocity (SV) can be used. This technique measures the rate of transport of the complex, which is determined by its size, density and shape [35]. However, more commonly the sedimentation equilibrium (SE) method is employed for dynamic associations and provides thermodynamic data on the protein-protein interaction [36]. The range of K_D measurable using AUC is approximately 10^{-4} - 10^{-8} .

1.4.2.4 Surface Plasmon Resonance (SPR)

The phenomenon of SPR can be used to measure binding events on metallic surfaces. SPR is an electron charge-density wave phenomenon, which arises at the surface of a metallic layer when light is reflected at the layer under conditions of total internal reflectance. The surface plasmons that are generated are sensitive to any changes in the refractive index of the medium on the opposite side of the metallic layer from the reflected light. Protein-protein interactions occurring at the surface affect the refractive index of the medium and can therefore be detected [37].

The technique requires only small amounts of material and both of the interacting biomolecules can be used in a label-free form. Experimental design is critical, however, as some features of the technology, and the fact that one of the proteins must be surfaced attached, can give rise to misleading results [19, 37, 38].

SPR is now widely used in industry to measure protein-protein interactions and provides information on the specificity, kinetics and affinity of the binding event [38, 39]. Recently advances in the technology, and in the software, have enabled the thermodynamics of protein-protein associations to be derived from the kinetic measurements [38, 40]. The technique can be used to measure K_D over the range 10^{-4} - 10^{-11} , association rate constants (k_a) between 10^3 and 10^7 and dissociation rate constants (k_d) between 10^{-1} and 10^{-6} .

1.4.2.5 Dual Polarisation Interferometry (DPI)

Another, very recent, surface-based protein-protein interaction measurement technique is that of dual polarisation interferometry (DPI). This technique utilises two stacked waveguides, a reference waveguide and a sensing waveguide that channel polarised light along their length. The split beam recombines after leaving the two waveguides to form an interference pattern [41]. The polarised light can produce an evanescent wave perpendicular to the waveguide or parallel to the waveguide, both of which can extend into the medium above the sensing waveguide. These fields are both affected by refractive index changes in the medium close to the surface but to differing extents. Any change in the refractive index at the surface, caused by the binding of material, results in changes to the interference pattern. This is because the refractive index change cause a change in the speed of the light travelling down the waveguide [41]. By using the information provided by both polarisations, and solving the Maxwell equation for both, the thickness and density of the layer on the surface of the waveguide can be determined [41]. The technology is able to measure thickness changes of $< 0.1 \text{ \AA}$ and density changes $< 0.1 \text{ pg}\cdot\text{mm}^{-2}$.

Gaining information on both the thickness and density of the layers at the waveguide surface can also enable conformational changes to be detected in the interacting proteins. While this technique is label free, the immobilisation of one of the proteins on the surface can lead to the same problems encountered with SPR.

1.4.2.6 Mass Spectrometry (MS)

The use of mass spectrometry for the identification of binding partners after immunoprecipitation or “tag” purification is now routine, although problems still persist with non-specific binding [42, 43]. Cross-linking [44], surface labelling [45] and hydrogen/deuterium (H/D) exchange [46] are all useful techniques for determining both interaction boundaries and the protein-protein interface [43]

The biophysical analysis of intact protein-protein interactions using electrospray ionisation MS is somewhat still in its infancy although examples do exist in the literature [47, 48]. The technique provides information on the stoichiometry of the complex and also the gas-phase dissociation energetics. However, the assumption is often made that the gas-phase complex is similar to the solution based one [43].

1.5 Selection Guidelines for Interaction Pairs

The interaction pairs were selected on the basis that they would be used across numerous measurement platforms including protein microarrays spotted with human protein libraries. Hence the selection criteria were designed with this end-point application in mind. The finalised selection criteria, along with reasons, are listed in Table 1.1. These criteria were used as decision gateways while searching the literature for suitable interaction pairs.

Selection Criteria	Reason
Within the dissociation constant (K_D) range 10^{-6} - 10^{-9}	Ideally want interaction pairs that cover this range of K_D values. Industry interested in weak binders as well as very strong binders.
Very well characterised	Important that the biophysical properties of the interaction pairs are well documented and that there is ideally a crystal structure of the complex
Simple expression and purification	Important as proteins were all to be produced recombinantly in-house, under tight time constraints
Low non-specific binding to other proteins	Important if used as standards in microarrays assays where they are exposed to a large number of different proteins
Non-human	Important if used with human antibody, cancer receptor, or cytokine libraries in order to minimise non-specific binding
Orthogonal assay to verify protein folding (e.g. enzyme assay)	Useful for verifying the proteins are natively folded

Table 1.1: The list of criteria used in selecting potential interaction pairs.

A number of specific interaction pairs were assessed in more depth before the final selection was made. These interaction pairs are listed in Table 1.2.

Interaction Pair	Reason for/against Selection	Reference
Interferon alpha2b / Interferon receptor	Purification of Receptor protein problematic	[49-53]
Bacterial Colicin E9 / Immunity Protein (Im9)	Difficult expression and purification Dissociation constant (K_D) to low (10^{-14} M)	[54-58]
<i>Serratia marcescens</i> haemophore HasA / haemoglobin	Complex purification	[59-64]
N-terminal domains of gene-3-protein (g3p) from M13 filamentous phage	Concerns over stability of protein domains Poorly defined binding parameters	[65-69]
<i>E. coli</i> Maltose binding protein sub-domains	Complex expression Poorly defined binding parameters	[70]
<i>Serratia marcescens</i> Metallo-protease (SMP) / Inhibitor from <i>Erwinia chrysanthemi</i> (Inh)	Non-specific activity of protease could cause problems	[71-73]
<i>E. coli</i> Tryptophan Synthase multi-subunit Enzyme	Suitable K_D Simple Purification Well Characterised (see Section 1.6.1)	[74-81]
<i>E. coli</i> N-utilisation Substance A / Phage Lambda Lambda N Antitermination Protein	Suitable K_D Simple Purification Well Characterised (see Section 1.6.2)	[82-87]

Table 1.2: List of interaction pairs scrutinised as possible candidates.

1.6 Interaction Pairs

1.6.1 *E. coli* Tryptophan Synthase – Multi-subunit Enzyme

The first interaction pair chosen was the multi-enzyme complex Tryptophan Synthase from *E. coli*. This protein complex is very well characterised and has been used previously as a model system for the study of protein-protein interactions [74]. It consists of two α -subunits (TrpA), which are present as monomers before complex formation, two β -subunits (TrpB), that are dimerised even in the absence of TrpA [75], and the co-factor pyridoxal-5'-phosphate (PLP) [81]. The protein subunits catalyse different reactions in the two stage synthesis of L-tryptophan from indole-3-glycerol phosphate [76]. While no crystal structure of the *E. coli* complex is available,

the structure of the *Salmonella typhimurium* complex (PDB code: 1BKS [77, 78]) has been used to predict interactions at the interface of the *E. coli* complex, as the amino acid sequences of the α and β -subunits from both species are very similar [76, 79, 80]. The crystal structure of the asymmetric unit ($\alpha\beta$) from *S. typhimurium* is shown in Figure 1.1.

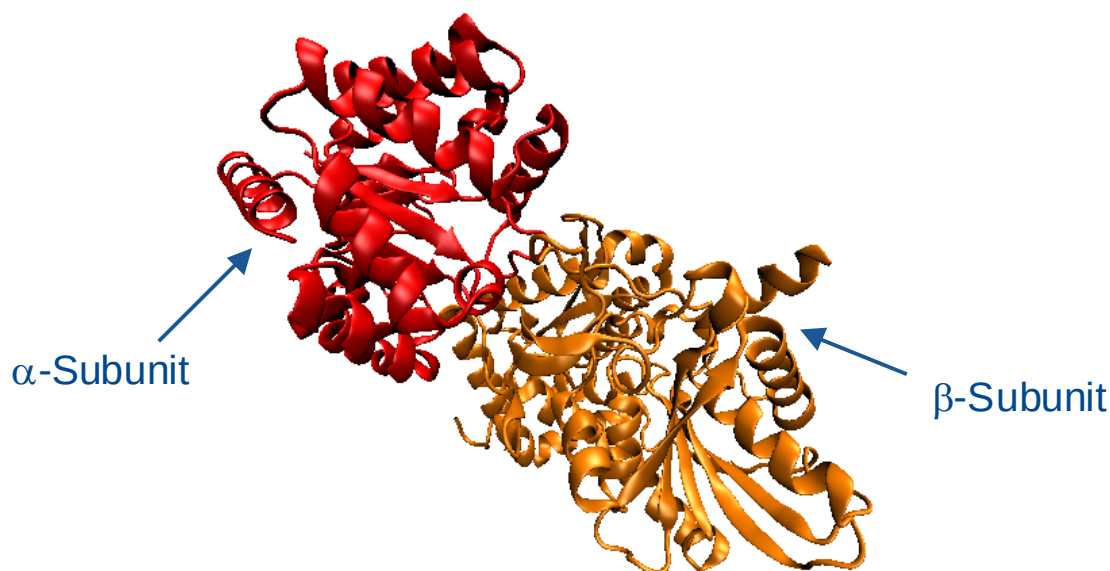


Figure 1.1: Crystal structure of Tryptophan Synthase from *S. typhimurium*. The asymmetric unit is shown (PDB code: 1BKS). The α and β -subunits are indicated.

The TrpA subunit contains 267 amino acids and has a predicted molecular weight of 28592.9 Da, and a theoretical pI is 5.31. The primary structure is shown in Figure 1.2. It can be expressed at high yield in *E. coli* and the purification is also relatively straightforward.

The TrpB subunit contains 396 amino acids and has a predicted molecular weight of 42852 Da, and has a theoretical pI of 5.71 – very similar to that of TrpA. Its primary structure is shown in Figure 1.2. As with TrpA expression and purification are reported to be relatively simple.

The binding affinity of the native complex was measured using ITC and found to be $5.32 \times 10^6 \cdot \text{M}^{-1}$ in the absence of salt [80]. Hiraga and Yutani have identified and characterised specific residues within the binding interface of the complex that formed hydrogen bonds during binding [80]. They showed that certain of these TrpA residues, when mutated to alanine, decreased the binding affinity of the complex by an order of magnitude [80]. By using these mutants it would be possible to prepare a number of interaction pair variants, with differing binding affinities, from a single ‘parent’ interaction pair.

A

<u>10</u>	<u>20</u>	<u>30</u>	<u>40</u>	<u>50</u>	<u>60</u>
ERYESLFAQL	KERKEGAFVP	FVTLGDPGIE	QSLKIIDTLI	EAGADALELG	IPFSDPLADG
<u>70</u>	<u>80</u>	<u>90</u>	<u>100</u>	<u>110</u>	<u>120</u>
PTIQNATLRA	FAAGVTPAQC	FEMLALIRQK	HPTIPIGLLM	YANLVFNKGI	DEFYAQCEKV
<u>130</u>	<u>140</u>	<u>150</u>	<u>160</u>	<u>170</u>	<u>180</u>
GVDSVLVADV	PVEESAPFRQ	AALRHNVAPI	FICPPNADD	LLRQIASYGR	GYTYLLSRAG
<u>190</u>	<u>200</u>	<u>210</u>	<u>220</u>	<u>230</u>	<u>240</u>
VTGAENRAAL	PLNHLVAKLK	EYNAAPPLQG	FGISAPDQVK	AAIDAGAAGA	ISGSAIVKII
<u>250</u>	<u>260</u>				
EQHINEPEKM	LAALKVFPVQP	MKAATRS			

B

<u>10</u>	<u>20</u>	<u>30</u>	<u>40</u>	<u>50</u>	<u>60</u>
TTLLNPYFGE	FGGMYVPQIL	MPALRQLEEA	FVSAQKDPEF	QAQFNDLLKN	YAGRPTALTK
<u>70</u>	<u>80</u>	<u>90</u>	<u>100</u>	<u>110</u>	<u>120</u>
CQNITAGTNT	TLYLKREDLL	HGGAHKTNQV	LGQALLAKRM	GKTEIIAETG	AGQHGVASAL
<u>130</u>	<u>140</u>	<u>150</u>	<u>160</u>	<u>170</u>	<u>180</u>
ASALLGLKCR	IYMGAKDVER	QSPNVFRMRL	MGAEVIPVHS	GSATLKDACN	EALRDWSGSY
<u>190</u>	<u>200</u>	<u>210</u>	<u>220</u>	<u>230</u>	<u>240</u>
ETAHYMLGTA	AGPHPYPTIV	REFQRMIGEE	TKAQILEREG	RLPDAVIACV	GGGSNAIGMF
<u>250</u>	<u>260</u>	<u>270</u>	<u>280</u>	<u>290</u>	<u>300</u>
ADFINETNVG	LIGVEPGGHG	IETGEHGAPL	KHGRVGIYFG	MKAPMMQTED	GQIEESYSIS
<u>310</u>	<u>320</u>	<u>330</u>	<u>340</u>	<u>350</u>	<u>360</u>
AGLDFPSVGP	QHAYLNSTGR	ADYVSITDDE	ALEAFKTLCL	HEGIIPALES	SHALAHALKM
<u>370</u>	<u>380</u>	<u>390</u>			
MRENPDKEOL	LVVNLSGRGD	KDIFTVHDIL	KARGEI		

Figure 1.2: Primary sequences of the (A) α -subunit and (B) β -subunit of *E. coli* Tryptophan Synthase

1.6.2 *E. coli* N-utilisation Substance A and Phage Lambda N Antitermination Protein

The second interaction pair chosen for this project comprised the *E. coli* N-utilisation substance A (NusA) and phage Lambda N antitermination protein (LamN). LamN is used by phage Lambda to regulate the transcriptional termination events during its life-cycle [82]. As part of this process it binds to RNA polymerase and the transcribed mRNA and then conscripts a number of N-utilisation substances, of which NusA is one, to form the final antitermination complex [83]. The interaction of LamN with NusA has been characterised in depth in the literature [85-87]. The interaction of the C-terminal region of NusA with the specific binding epitope in LamN [84] has also been characterised by Bonin *et al* [84].

Full-length NusA from *E. coli* contains 495 amino acids and has a molecular weight of 54871 Da, and a theoretical pI of 4.53. The C-terminal fragment used in the Bonin *et al* study comprises the two acidic repeats, AR1 and AR2 (350-495) and contains 145 amino acids. Its molecular weight is predicted to be 15872.6 Da with a theoretical pI of 3.97. The primary structure of full-length NusA along with the C-terminal acidic repeats is shown in Figure 1.3. Full-length LamN contains 106 amino acids, has a predicted molecular weight of 12167 Da, and a theoretical pI of 11.00.

Biophysical analysis of the complex between full-length NusA and full-length LamN determined that the complex has a K_D of 70×10^{-9} M [87]. Bonin *et al* determined the K_D of the interaction between the C-terminal acidic repeats of NusA (NusA Cterm) (Figure 1.3) and a LamN peptide (Figure 1.4) to be 3.55×10^{-6} M [84]. The crystal structure of the latter complex has been determined to a resolution of 1.9 Å and is shown in Figure 1.5. The critical binding contacts between the peptide and NusA Cterm have also been analysed using alanine scanning mutagenesis [84]. As with the TrpA/TrpB interaction pair, these mutational studies provide useful information for the construction of further interaction pair variants based on the initial native NusA Cterm/LamN peptide pair.

The purification and expression of NusA Cterm was reported as being straight forward [84], while the LamN peptide could be obtained commercially.

10	20	30	40	50	60
NKEILAVVEA	VSNEKALPRE	KIFEALESAL	ATATKKKYEQ	EIDVRVQIDR	KSGDFDTFRR
70	80	90	100	110	120
WLVVDEVTQP	TKEITLEAAR	YEDESLNLGD	YVEDQIESVT	FDRITTQTAK	QVIVQKVREA
130	140	150	160	170	180
ERAMVVDQFR	EHEGEIITGV	VKKVNRDNIS	LDLGNNAEAV	ILREDMLPRE	NFRPGDRVRG
190	200	210	220	230	240
VLYSVRPEAR	GAQLFVTRSK	PEMLIELFRI	EVPEIGEEVI	EIKAAARDPG	SRAKIAVKTN
250	260	270	280	290	300
DKRIDPVGAC	VGMRGARVQA	VSTELGGERI	DIVLWDDNPA	QFVINAMAPA	DVASIVVDED
310	320	330	340	350	360
KHTMDIAVEA	GNLAQAIGRN	GQNVRLASQL	SGWELNVMTV	DDLQAKHQA	E AHAIDTFTK
370	380	390	400	410	420
YLDIDEDFAT	VLVEEGFSTL	EELAYVPMKE	LLEIEGLDEP	TVEALRERAK	NALATIAQAQ
430	440	450	460	470	480
EESLGDNKPA	DDLLNLEGVD	RDLAFKLAAR	GVCTLEDLAE	QGIDDLADIE	GLTDEKAGAL
490					
IMAARNICWF	GDEA				

Figure 1.3: Primary sequence of NusA from *E. coli*. The C-terminal region designated as NusA Cterm is highlighted in yellow.

10	20	30	40	50	60
DAQTRRRERR	AEKQAQWKAA	NPLLVGVSAA	PVNRPILSLN	RKPKSR	VESA LNPIDLTVLA
70	80	90	100		
EYHKQIESNL	QRIERKNQRT	WYSKPGERGI	TCSGRQKIKG	KSIIPI	

Figure 1.4: Primary sequence of LamN from Phage Lambda. The epitope corresponding to the LamN peptide is highlighted in blue.

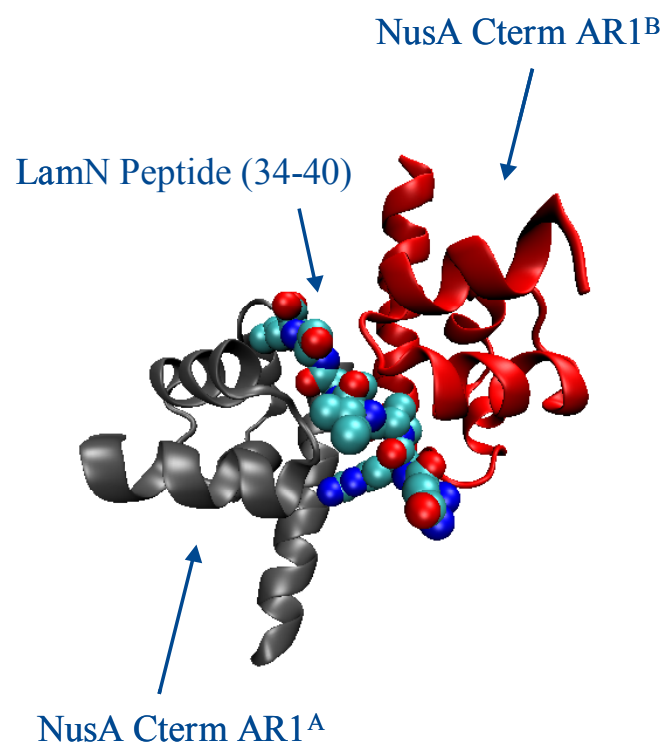


Figure 1.5: Crystal structure of NusA Cterm and LamN Peptide (PDB code: 1U9L). Two units of NusA Cterm were seen to bind to the LamN peptide in the crystal structure and these are indicated using the superscripts 'A' and 'B'. The AR2 region of NusA Cterm was not seen in the structure as it did not form an ordered structure [84].

2 Materials and Methods

2.1 Materials

All general chemicals were reagent grade unless otherwise stated and purchased from Sigma-Aldrich or Fisher Chemicals.

The restriction enzymes, NdeI and XhoI were purchased from New England Biolabs, USA (NEB):

All buffers and solutions were made up in ultrapure de-ionised water (18.2 MΩ) purified through a Millipore MilliQ synthesis A10 system.

All molecular biology methods are based on the protocols of Sambrook *et al* [88] unless otherwise stated

2.2 Cloning of Constructs

The authentic coding sequences for TrpA, TrpB and NusA cterm were prepared as follows. Genomic DNA was extracted from *E. coli* K12 DH5α cells using the PureLink™ Genomic DNA Purification kit (Invitrogen) and the integrity analysed with ethidium bromide 1% agarose gel electrophoresis. The purified genomic DNA was used as the template in polymerase chain reactions (PCR) using Pfu Ultra HS polymerase (Stratagene) as per the manufacturer's instructions, and the primers listed below:

TrpA isolation primers

Sense: 5'-ATGGAACGCTACGAATCTC-3'

Anti-sense: 5'-TTAACTGCGCGTCGC-3'

TrpB isolation primers

Sense: 5'-ATGACAACATTACTTAACCCCT-3'

Anti-sense: 5'-TCAGATTTCCTCGTG-3'

NusA Cterm isolation primers

Sense: 5'-GAAGCGCACGCAGC-3'

Anti-sense: 5'-TTACGCTTCGTCACCGA-3'

Following the PCR reactions, products were separated using 2% agarose gel electrophoresis and the correctly sized PCR fragments were purified using a QIAquick Gel Extraction Kit (QIAGEN). The correct product sizes were as follows: TrpA = 807 bp, TrpB = 1194 bp and NusA Cterm = 438 bp.

Modified DNA coding sequences of the three proteins containing unique restriction sites were prepared as set out below. PCR reactions were designed to add an NdeI restriction site to the 5' end and an XhoI site to the 3' end of the amplified PCR fragments using the following primers (the restriction sites are underlined):

TrpA modification primers

Sense: 5'-GACCTGACCATATGGGAACGCTACGAATCTC-3'

Anti-sense: 5'-CTACTCGAGTTAACTGCGCGTCGC-3'

TrpB modification primers

Sense: 5'-GACCTGACCATATGACAACATTACTTAACCCCT-3'

Anti-sense: 5'-CTACTCGAGTCAGATTTCCCCTCGTG-3'

NusA Cterm modification primers

Sense: 5'-GACCTGACCATATGGGAAGCGCACGCA-3'

Anti-sense: 5'-CTACTCGAGTTACGCTTCGTCACCGA-3'

Correctly sized PCR products were identified and purified as before.

PCR products were cut using NdeI (20 U) and XhoI (20 U) restriction enzymes in NEBuffer 4 (NEB) for 3 hours at 37 °C. The cut DNA fragments were purified and ligated into a pET-21b vector (Novagen), linearised with NdeI and XhoI, using the Quick Ligation™ kit (NEB). Competent *E. coli* DH5α cells were transformed with the ligated constructs and streaked out on LB agar plates containing 100 µg·ml⁻¹ ampicillin after 1 hour of incubation at 37 °C and 200 rpm.

Colonies were selected, grown and screened for the inserts with PCR using the universal pET primers, sense: 5'-ATCTCGATCCCGCGAAATTAATAC-3' and anti-sense: 5'-TTAGCAGCCGGATCTCAGT-3'. Constructs found to contain the correct insert (TrpA = 807 bp, TrpB = 1194 bp and NusA Cterm = 438 bp) were purified using the FastPlasmid Mini Kit (Eppendorf).

Constructs were sequenced at MWG-BIOTECH AG, (Germany) using standard T7F and T7R primers as well as the primers shown below which were complementary to internal sequences within each construct. These additional sequencing primers were necessary as the cDNA regions of the constructs were too long to be completely sequenced using only the T7F and T7R primers.

TrpA sequencing primers

Sense: 5'-GATTCGCCAGAAACACCCG-3'

TrpB sequencing primers

Sense: 5'-CCAAAGACGTTGAACGCCAGT-3'

Anti-sense: 5'-GCTCCACACCAATCAGGC-3'

NusA Cterm sequencing primers

Sense: 5'-CAGAAAGTGCGTGAAGCCG-3'

Anti-sense: 5'-CAGGATCACGGCTTCAGC-3'

2.3 Expression and Purification of Proteins

All recombinant proteins were expressed in *E. coli* BL21 (DE3). Competent BL21 (DE3) cells (Novagen) were transformed with the appropriate construct in pET-21b using heat shock, and streaked out on LB agar plates containing 100 $\mu\text{g}\cdot\text{ml}^{-1}$ ampicillin after 1 hour of incubation at 37 °C and 200 rpm. A single healthy colony was picked after overnight incubation at 37 °C and used to inoculate 10 ml LB broth containing 100 $\mu\text{g}\cdot\text{ml}^{-1}$ ampicillin, which were then incubated overnight at 37 °C and 200 rpm. Overnight cultures were used to inoculate 750 ml LB broth containing 100 $\mu\text{g}\cdot\text{ml}^{-1}$ ampicillin, these were incubated at 37 °C and 200 rpm until log phase growth had been achieved ($\text{OD}_{600} = 0.6-0.9$). Over-expression of the recombinant proteins was under the control of an isopropyl β -D-1-thiogalactopyranoside (IPTG) inducible promoter. Protein expression was initialised by addition of IPTG to a final concentration of 1 mM, and allowed to continue for 3 hours at 37 °C and 200 rpm. Cells were harvested by centrifugation ($3000 \times g$, 20 minutes at 4 °C) and the pellets stored at -20 °C.

2.3.1 Protein Extraction

All expressed proteins were extracted from the *E. coli* cell pellet using the same procedure. The only difference was the lysis buffer components for each protein. Below are listed the lysis buffers.

TrpA: 20 mM potassium phosphate pH 8.0, 1 mM EDTA

TrpB: 20 mM potassium phosphate pH 8.0, 1 mM EDTA, 0.02 mM pyridoxal-5'-phosphate (PLP)

NusA Cterm: 25 mM potassium phosphate pH 8.0, 1 mM EDTA

In each case, the *E. coli* cell pellet was thawed on ice and resuspended in lysis buffer (3 $\text{ml}\cdot\text{g}^{-1}$ wet pellet). Lysozyme (133 μl of a 6 $\text{mg}\cdot\text{ml}^{-1}$ stock per gram of wet pellet) and 1 tablet of complete™ Mini, EDTA-free anti-protease cocktail (Roche Applied Science) per 10 ml of lysis solution were added to the resuspended pellet, and the solution was incubated at room temperature for 30 minutes. The lysis mixture was subjected to 3 to 5 freeze-thaw cycles until it became viscous whereupon DNase I was added (to a final concentration of 20 $\mu\text{g}\cdot\text{g}^{-1}$ of wet pellet). The lysate solution was then incubated at room temperature until the solution was no longer viscous. The lysate was subsequently sonicated for 5 minutes on ice (25% amplitude, pulse: 9 seconds on, 1 second off) (Sonics Vibra Cell™) and then centrifuged ($75000 \times g$, 25

minutes at 4 °C). The supernatant was retained and filtered through a 0.22 µm filter to remove any unwanted cell debris.

2.3.2 Purification of TrpA

The purification of TrpA was adapted from Yutani *et al* [89].

Ammonium sulphate was slowly added with stirring (17.2 g per 100 ml of lysate solution) to the filtered lysate solution at 4 °C and allowed to equilibrate with stirring for 60 minutes at the same temperature. Precipitated material was pelleted by centrifugation (75000 × g, for 60 minutes at 4 °C). The supernatant was retained and ammonium sulphate (26.3 g per 100 ml solution) added again at 4 °C with stirring. The precipitated material was collected by centrifugation as before and the pellet was retained. This was dissolved in 20 mM potassium phosphate pH 7.0 (20 ml) and then dialysed against 5 mM potassium phosphate pH 7.0 (1000 ml) overnight at 4 °C.

The protein was further purified on a 5 ml HiTrap DEAE FF column (GE Healthcare) equilibrated in 5 mM potassium phosphate pH 7.0 at 4 °C. The column was developed at 2.5 ml·min⁻¹, and the protein of interest was eluted at ~ 20 mM potassium phosphate using a 20 column volume (CV) linear gradient of 5-100 mM potassium phosphate pH 7.0 (Figure A.1) The product identity was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and when stained with Coomassie brilliant blue G-250 a single band at the expected molecular weight of ~ 28.7 kDa was observed.

2.3.3 Purification of TrpB

The purification of TrpB was modified from Ogasahara *et al* [90].

Ammonium sulphate was slowly added with stirring (5.6 g per 100 ml of lysate solution) to the filtered lysate solution at 4 °C and allowed to equilibrate with stirring for 60 minutes at the same temperature. Precipitated material was pelleted by centrifugation (75000 × g, for 60 minutes at 4 °C). The supernatant was retained and ammonium sulphate (18.7 g per 100 ml solution) added again with stirring at 4 °C. The precipitated material was collected by centrifugation as before and the pellet was retained. This was dissolved in 500 mM Tris-HCl pH 8.0 (20 ml) and then dialysed against 100 mM potassium phosphate pH 7.0, 1 mM dithiothreitol (DTT), 5 mM EDTA, 0.02 mM PLP (1000 ml) overnight at 4 °C.

Further purification was carried out using a 5 ml HiTrap DEAE FF column (GE Healthcare) equilibrated in 100 mM potassium phosphate pH 7.0, 1 mM DTT, 5 mM EDTA, 0.02 mM PLP at 4 °C. The column was developed at a flow rate of 2.5 ml·min⁻¹ and TrpB was eluted at ~ 185 mM NaCl in a 20 CV linear gradient of starting buffer containing 0-500 mM NaCl (Figure A.2). The product identity was confirmed by SDS-PAGE and when stained with coomassie blue all fractions that contained a single band at the expected molecular weight of ~ 42.8 kDa for TrpB were pooled. Pooled fractions were concentrated using a 10 kDa Mwco Vivaspin centrifugal concentrator (Sartorius), filtered through a 0.22 µm filter and applied to a Superdex 75 16/60 column (GE Healthcare) equilibrated in 50 mM potassium phosphate pH 7.0, 300 mM KCl, 0.1 mM DTT, 5 mM EDTA, 0.02 mM PLP. This column was developed at 0.5 ml·min⁻¹ and the main peak eluted after ~ 57 ml. This peak contained a shoulder, so fractions corresponding to the main peak and the shoulder were pooled separately (Figure A.3). For both the shoulder and main peak

fractions, product identity was confirmed by SDS-PAGE. Coomassie brilliant blue G-250 stained fractions contained a single band at ~ 42.8 kDa – the expected molecular weight of TrpB (see Section 3.1.1.2).

2.3.4 Purification of NusA Cterm

The filtered lysate was applied to a 5 ml HiTrap Q FF column (GE Healthcare) equilibrated in 5 mM potassium phosphate pH 7.0 and developed at a flow rate of 2 ml·min⁻¹. The protein of interest was eluted at ~ 880 mM potassium phosphate using a 10 CV linear gradient of 0.005-1 M potassium phosphate pH 7.0 (Figure A.4). The product identity was confirmed by SDS-PAGE and when stained with coomassie blue a single band at the expected molecular weight of NusA Cterm (15.8 kDa) was observed. Pooled fractions were concentrated using a 3 kDa, Mwco Vivaspin centrifugal concentrator (Sartorius) and the concentrated protein was filtered through a 0.22 µm filter. The protein was then applied to a Superdex 75 16/60 column (GE Healthcare) equilibrated in 50 mM Tris pH 7.0, 150 mM NaCl, 2 mM DTT and developed at 1 ml·min⁻¹. The protein eluted as a single peak after ~ 70 ml (Figure A.5) and the product identity was confirmed by SDS-PAGE. After staining with Coomassie brilliant blue G-250, a single band was observed at the expected molecular weight of 15.8 kDa.

2.3.5 Protein Concentration Measurements

All protein concentration measurements were carried out using a Perkin Elmer Lambda 850 UV/VIS spectrometer. Protein concentration was measured using UV/VIS spectrometry scanning from 240 nm to 350 nm. The average absorbance at either 278 nm or 280 nm from three separate measurements was used to calculate the concentration of the protein material using Equation 2.1.

Equation 2.1 $\epsilon_{\text{percent}} cL / 10 = A$

Where $\epsilon_{\text{percent}}$ = the absorbance of 10 mg·ml⁻¹ of protein at stated wavelength in a 1 cm pathlength cell

c = concentration (mg·ml⁻¹),

L = pathlength (cm),

A = absorbance at stated wavelength

For TrpA $\epsilon_{\text{percent}} = 4.4$ at 278 nm [91, 92]

For TrpB $\epsilon_{\text{percent}} = 6.5$ at 280 nm [75]

For NusA Cterm $\epsilon_{\text{percent}} = 5.42$ at 280 nm [93]

2.3.6 Preparation of the LamN Peptide

The amino acid sequence highlighted in Figure 1.4 shows the LamN peptide sequence used for this study. The LamN peptide was synthesized by Sigma Genosys and shipped as a lyophilised powder. To enable simple immobilisation of the peptide in

any surface-based measurement, the N-terminus of the peptide was biotinylated. An alanine residue was added to the N-terminus in order to improve the biotinylation reaction. Hence the total number of residues in the peptide was 15. The linker used was aminohexanoic acid and this introduced a 6 carbon-spacer between the peptide and the biotin molecule. The supplier determined the peptide's molecular weight to be 2087 Da using MALDI-TOF MS. The purity was shown to be 95% using reverse phase chromatography. The primary sequence of the peptide is shown below:

Biotin-ANRPILSLNRKPKSR

The peptide was dissolved in ultra pure water, flash-frozen on dry ice and then storage at -80°C . The frozen peptide was lyophilised again overnight and then stored at -20°C until used.

2.3.7 Preparation of Interaction Pairs for Binding Measurements

For both the ITC and SPR measurements it was critical that the respective components of an interaction pair were in identical buffers. For the proteins this was achieved by repeated ($3 \times$) dialysis at 4°C against the relevant buffer (1000 ml) (see below). After dialysis the protein concentrations were measured using the method described in Section 2.3.5. SDS PAGE analysis was used to check the integrity and purity of the proteins, (Figure 2.1).

The LamN peptide was too small to be dialysed, so the lyophilised powder was weighed out and dissolved in the same buffer used for the dialysis of NusA Cterm. Because the peptide contained no spectroscopically active amino acids it was not possible to measure the concentration as outlined in Section 2.3.5. Every effort was made to ensure the accuracy of the gravimetric measurements. The weighing balance (GENIUS Sartorius) used was accurate 0.1 mg and a volumetric flask accurate to 0.25 ml was used for measuring the buffer volume.

The following buffers were used for all binding measurements and were the buffers the proteins were dialysed against:

TrpA/TrpB buffer	50 mM potassium phosphate pH 7.0, 300 mM KCl, 0.1 mM DTT, 5 mM EDTA, 0.02 mM PLP
------------------	--

NusA Cterm/LamN Peptide buffer	50 mM Tris pH 7.0, 150 mM NaCl, 2 mM DTT
--------------------------------	--

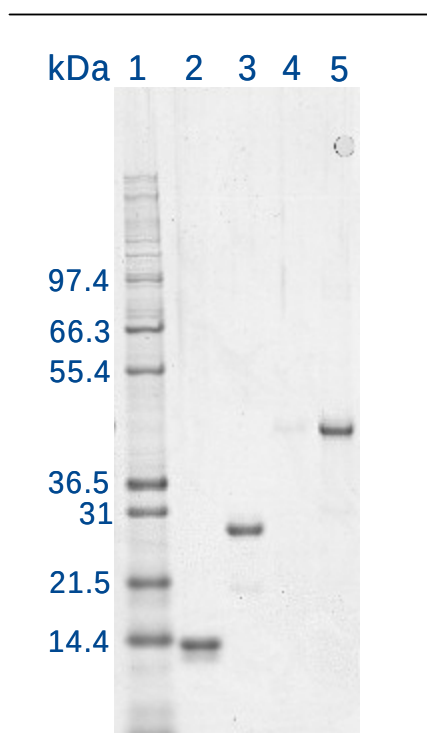


Figure 2.1: 12% SDS PAGE analysis of the interaction pair proteins after final preparation. Lane 1: Marker; Lane 2: NusA Cterm; Lane 3: TrpA; Lane 4: Empty; Lane 5: TrpB.

2.4 Isothermal Titration Calorimetry (ITC) Binding Studies

Throughout this section ‘macromolecule’ refers to the species present in the instrument cell while ‘ligand’ refers to the species present in the injection syringe. The proteins chosen to be the macromolecule and those to be the ligand are shown in Table 2.1. All solutions and the instrument cell were equilibrated at the correct run temperature prior to measurement. To ensure that bubbles did not form in either the macromolecule or ligand solutions during the measurements, all solutions were degassed before loading using a ThermoVac (MicroCal Inc).

Interaction Pair	Macromolecule	Ligand
TrpA/TrpB	TrpB	TrpA
NusA Cterm/LamN Peptide	NusA Cterm	LamN Peptide

Table 2.1: Assignment of the macromolecule and ligand for both interaction pairs.

2.4.1 Instruments

For the ITC experiments three separate VP-ITC (MicroCal Inc) instruments were used. The VP-ITC instruments were all operated using VPViewer 2000 software (MicroCal Inc).

2.4.2 Control Experiments

Before each measurement two control experiments were carried out to check for unwanted sources of heat effects. The first control experiment comprised a water-water baseline measurement. This control assessed contributions to heat effects from the instrument itself due to, for instance, contamination of the instrument cell. The second control experiment involved injecting ligand into buffer containing no macromolecule. This control examined the heats of dilution, which can arise from buffer differences and ligand/ligand binding events. These heats should be negligible and can be deleted from the actual measurement data if believed to be significant. Due to the careful steps taken in preparing the samples (Section 2.3.7) no significant heats of dilution were observed for any of the ITC measurements. For this reason it was not necessary to remove these control heats from the experimental data.

2.4.3 Interaction Pair Binding Measurements

The experimental parameters for each interaction pair are listed in Table 2.2 and were chosen after analysis of Higara and Yutani [80] for the TrpA/TrpB pair and Bonin *et al* [84] for the NusA Cterm/LamN peptide pair. For all ITC measurements a single low volume injection (set at 3 μ l) was made before the other injections. This was carried out in order to minimise the possibility of the first injection showing a smaller heat effect than expected due to leakage of ligand from the syringe. This control data point was deleted from the binding isoforms before fitting the data. The cell and syringe were loaded as per the manufacturer's instructions.

After each measurement the cell was thoroughly cleaned 3 $\times$ using ultrapure de-ionised water (18.2 M Ω).

Parameter	Interaction Pair	
	TrpA/TrpB	NusA Cterm/LamN Peptide
Number of Injections	17 or 18	17 or 18
Run Temperature	40 °C	25 °C
Reference Power	10 $\mu\text{cal}\cdot\text{sec}^{-1}$	10 $\mu\text{cal}\cdot\text{sec}^{-1}$
Initial Delay	60 sec	60 sec
Syringe Concentration	0.05 mM	0.3-0.35 mM
Cell Concentration	0.01 mM	0.03 mM
Stirring speed	300 rpm	300 rpm
Injection Volume	15 μl	15 μl
Injection Duration	30 sec	30 sec
Injection Spacing	240 sec	240 sec
Filter Period	2 sec	2 sec

Table 2.2: List of the experimental parameters used for the ITC measurements.

2.4.4 Analysis of the Thermal Titration Data

All data analysis was carried using Origin® v7 software (OriginLab Corp). The built in non-linear least squares (NLLS) fitting model for **One Set of Sites** was used for all curve fitting based on the previous analyses [80, 84]. Repeated iterations were carried out until the reduced χ^2 value no longer decreased. The One Set of Sites model has three separate parameters; these are N (stoichiometry), K_A (association binding constant in M^{-1}) and ΔH (heat change in $\text{cal}\cdot\text{mol}^{-1}$). The fourth parameter ΔS (change in entropy in $\text{cal}\cdot\text{K}^{-1}\cdot\text{mol}^{-1}$) is calculated from K_A and ΔH using Equation 2.2 and Equation 2.3.

Equation 2.2 $\Delta G = -RT \ln K_A$

Equation 2.3 $\Delta G = \Delta H - T\Delta S$

Where ΔG = the Gibbs free energy
 R = the Gas constant
 T = the measurement temperature in Kelvin

2.5 Surface Plasmon Resonance (SPR) Binding Studies

Throughout this section ‘ligand’ refers to the protein immobilised to the surface, and ‘analyte’ to the protein in solution.

2.5.1 Instrument

SPR binding studies were performed on a T100 Biacore using Biacore T100 Evaluation Software V1.1 (Uppsala, Sweden).

2.5.2 Materials

All reagents were purchased from Biacore specifically for use in the T100 unless otherwise stated. For maleimide-coupling ethylenediamine (Puriss for GC, purity $\geq 99.8\%$) was purchased from Fluka and *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (Sulfo-MBS) was purchased from Pierce (Rockford, IL). To reduce non-specific binding (NSB) in the flow system of the Biacore, buffers are often supplemented with the surfactant P20. For Biacore studies both TrpA/TrpB and NusA Cterm/LamN peptide buffers (Section 2.3.7) were supplemented with 0.005% v/v P20 unless otherwise stated. Two commercially available chips were used for the SPR binding measurements, the standard CM5 chip, which contains a dextran layer, and the SA chip, which contains a layer of streptavidin covalently immobilised on the dextran surface.

2.5.3 Amine Coupling

The protocol for amine coupling was performed as directed by Biacore [37], and unless otherwise stated, all flow rates were $10 \mu\text{l}\cdot\text{min}^{-1}$. A brief summary of the steps are shown below:

1. Typically, amine coupling was performed in HBS-EP buffer unless stated. On the bare chip NSB of a saturating analyte concentration was analysed using a 120 seconds injection at $30 \mu\text{l}\cdot\text{min}^{-1}$ in the appropriate assay buffer. Any NSB was removed with a 60 seconds wash of 10 mM NaOH.
2. Pre-concentration on the chip was performed using low ionic strength buffers, with selection based on the pI of the ligand and surface. Typically $10\text{--}50 \mu\text{g}\cdot\text{ml}^{-1}$ of ligand was diluted into the appropriate buffer and applied onto the chip for 120 seconds to observe pre-concentration of the ligand. Before activation the chip was washed with 60 seconds flushes of 10 mM NaOH and 5 M NaCl to remove any protein non-covalently bound to the chip.
3. The CM5 chip surface was activated using a 0.4 M (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC)/0.1 M *N*-hydroxysuccinimide (NHS) (1:1) that had been prepared fresh. An EDC/NHS mix (600 μl) was

injected over the surface of the chip; typically this caused an increase of ~ 300 response units (RU).

4. The ligand was prepared at concentrations between 1-50 $\mu\text{g}\cdot\text{ml}^{-1}$ in the optimum immobilisation buffer determined in step 2. Ligand was then injected over the surface until the required amount of immobilisation had occurred (the required ligand level ideally gave an R_{max} of 100-200 RU upon steady-state binding, this was determined using Equation 2.4).
5. The CM5 chip was blocked using 1 M ethanolamine-HCl, pH 8.5; typically two, 60 seconds injections deactivated the chip and resulted in a stable baseline.
6. The relative activity of the surface was determined by injecting a saturating amount of analyte over the surface so steady state binding was achieved. At steady state the percentage occupancy of the chip can be determined using Equation 2.4. The theoretical R_{max} value can be calculated. Where the theoretical R_{max} deviates from that observed experimentally it indicates that there is either lower surface activity and/or a different stoichiometry of binding present in the system.

Equation 2.4
$$R_{\text{max}} = \left(\frac{\text{analyteMW}}{\text{ligandMW}} \right) \times R_L \times S_m$$

R_L = immobilization level

S_m = stoichiometric ratio

2.5.4 Preparation of the Maleimide Surface and Thiol Coupling

The maleimide surface was used to facilitate covalent binding of protein to the chip surface through reduced cysteine residues within the ligand. The method to prepare the maleimide surface was adapted from the Biacore Sensors Surface Handbook [37]:

1. The first stage in preparing a maleimide surface was conducted outside the Biacore to facilitate longer reaction times when converting the dextran from carboxyl to amine reactive groups. The surface of the CM5 chip was washed with distilled water and dried using a stream of nitrogen.
2. A mixture of 0.4 M EDC/0.1 M NHS (1:1) was freshly prepared, and 50 μl of this solution added to the CM5 chip surface. This was then incubated in a humidified environment for 45 minutes.
3. The chip was washed and dried as above, 50 μl of a 0.2 M solution of ethylenediamine, prepared in 100 mM sodium borate buffer pH 8.5 was added to the chip surface and incubated overnight in a humidified environment.
4. The chip was washed as above and deactivated with 20 mM NaOH for 20 minutes, then washed again and dried.
5. The chip was inserted into its protective case and inserted into the Biacore; further modification/analysis were performed in HBS-EP buffer unless otherwise stated. All 4 flow-cells (Fc) were modified unless otherwise stated.

6. Maleimide functional groups were introduced by injecting 50 mM Sulfo-MBS prepared in 100 mM sodium borate pH 8.5 for 600 seconds at $10 \mu\text{l}\cdot\text{min}^{-1}$ over the sensor surface. Typically this resulted in an increase of ~ 3000 RU.
7. To avoid oxidation of the reactive cysteine residues, the ligand was buffer-exchanged into DTT-free buffers immediately before immobilisation to the chip. Buffer exchange was performed in centrifugal filter columns (regenerated cellulose) from Millipore (MA, USA) using the appropriate molecular weight cut-off. Centrifugation was performed at 4°C and repeated until the molar ratio of DTT was at least 100-fold less than the number of cysteine in the ligand (DTT contains 2 reactive thiol moieties).
8. The ligand was prepared at concentrations between $1\text{-}50 \mu\text{g}\cdot\text{ml}^{-1}$ in the appropriate immobilisation buffer. Ligand was injected at $10 \mu\text{l}\cdot\text{min}^{-1}$ until the required amount of immobilisation had occurred (Equation 2.4).
9. The maleimide surface was blocked using a cysteine/NaCl solution (50 mM cysteine and 1 M NaCl prepared in 100 mM sodium acetate, pH 4.0) injected for 240 seconds at $10 \mu\text{l}\cdot\text{min}^{-1}$.

2.5.5 Blank 'Reference' Flow-Cell

Unless stated Flow-cell1 (Fc1) was employed as the reference flow-cell. The reference flow-cell was prepared as detailed in Sections 2.5.3, or 2.5.4 except no protein was added to the surface, therefore after activation with EDC/NHS the surface was immediately blocked. Ideally a reference surface would contain a non-active variant of the ligand, immobilised at a similar density, therefore providing the best reference to compensate for changes in refractive index, or bulk effects. However, within the literature there were no examples of proteins that had been shown to act as suitable inactive models of TrpA, TrpB, or NusA Cterm.

Unless stated all the sensograms and kinetic experiments had the reference 'Fc1' data subtracted from them to remove measurement artefacts resulting from refractive index changes and bulk effects.

2.5.6 Regeneration Buffer Scouting

Regeneration Scouting was performed using the Biacore wizard program. Solutions tested included those within the Biacore Regeneration Scouting Kit (BR-1005-56) and custom made solutions specific for the interaction model. Typically a single injection for 30 seconds of the regeneration solution was attempted with three sequential repeats so trends could be observed. After the ideal regeneration solution was determined further optimisation was performed in terms of time, concentration and number of injections. The regeneration solutions analysed for all chips were typically run in this order:

1. 4 M MgCl_2
2. 5 M NaCl
3. 10 mM Glycine_HCl, pH 3.0
4. 10 mM Glycine_HCl, pH 2.5
5. 10 mM Glycine_HCl, pH 2.0
6. 10 mM Glycine_HCl, pH 1.5

7. 0.5% SDS
8. 100% Ethylene Glycol
9. 10% v/v P20
10. 10 mM NaOH
11. 200 mM NaOH

2.5.7 Control Reactions

To validate a chip surface, typically, three control experiments are performed: surface performance, linked reactions and mass transfer limitation. Surface performance tests the stability of the surface by analysing the baseline stability and total binding from a series of up to 10 binding/regeneration cycles. Linked reactions describe a model in which in a time dependent manner binding of the analyte is followed by a conformational change in the ligand/analyte complex, resulting in a stabilisation of this complex and a change in the dissociation rate. Mass transport limitation occurs when the rate of transport of the analyte to and from the chip surface becomes the rate-limiting step for the overall ligand/analyte interaction.

2.5.8 Kinetic/Affinity Analysis

Kinetic and affinity experiments were run at $30 \mu\text{l}\cdot\text{min}^{-1}$ at either 25°C or 40°C for NusA Cterm/LamN peptide and TrpA/TrpB respectively. Before performing the kinetic experiment, the Biacore was primed to remove any residual traces of previous buffers and the detector was normalised. To condition/stabilise the chip, 3-5 start-up binding cycles were performed using a mid-range analyte concentration. For kinetic experiments, 8 dilutions of the analyte and two zero analyte runs were performed – to determine repeatability a single non-zero concentration of analyte was included at the end of all the cycles. For affinity analysis, samples were run twice in a randomised format.

3 Results and Discussion

A number of interaction measurement platforms were reviewed in Section 1.4. Of these, isothermal titration calorimetry (ITC) and surface plasmon resonance (SPR) were used in this study. Both of these techniques are well established within the biopharmaceutical industry and used across the drug discovery and development process.

There are a few specific studies in the literature which compare binding data collected using these two techniques, however no systematic comparisons have been reported using protein-protein interaction pairs. The studies currently available include the interaction of heparin with heparin binding growth-associated molecule (HB-GAM) [94]; interaction of the peptide polymyxin B with endotoxin [95]; interaction of carbonic anhydrase II with the inhibitors 4-carboxybenzenesulfonamide (CBS) and 5-dimethyl-amino-1-naphthalene-sulfonamide (DNSA) [96]; interaction of cAMP-dependent protein kinase (PKA) with cAMP [97]; interaction of a dimeric derivative of vancomycin to L-Lys-D-Ala-D-lactate [98] and the interaction of the anti-cell death protein BAG-1 with a 70 kDa heat shock chaperone (Hsc70) [99]. Of these studies, [96], [95] and [97] found the two techniques gave similar values for the K_D while the following studies reported K_D values with poor agreement [94], [98] and [99]. Interestingly Moll *et al* found that while the dissociation constant for the PKA/cAMP interaction measured using the two techniques was similar, the underlying thermodynamics were not [97]. Towards the end of this project, an important follow-on study was published, based on the work reported in [96]. The new study involved 22 participants and focused on the characterisation, using SPR and ITC, of the thermodynamics of interaction of four sulfonamide-based inhibitor molecules and carbonic anhydrase II [100]. The researchers measured the inhibitor-protein interactions at multiple temperatures and used van't Hoff plots to derive the enthalpies and entropies of the interactions. For this system the thermodynamic parameters calculated from the SPR measurements matched those determined using ITC. This study makes an important contribution to understanding the measurement issues involved in using SPR to analyse the thermodynamics of interactions, but it uses a protein-small-molecule interaction pair, rather than a protein-protein interaction pair, as the model system. Due to their greater structural complexity, protein-protein interaction pairs are, in general, more difficult to use as model systems for comparing in-solution and surface binding, than their protein-small-molecule counterparts. There is a need, however, for a systematic study, similar to the one mentioned above, that utilises protein-protein interaction pairs. The results shown here represent a step towards addressing that need.

3.1 Isothermal Titration Calorimetry (ITC)

The binding parameters of both interaction pairs were assessed using ITC over a period of 10 months. This was done in order to determine the stability of the interaction pairs, as this characteristic is very important for their successful application as inter-comparison materials. This measurement period was chosen to begin once protein purification had been completed and protein storage had commenced. The interaction pairs were stored at $-80\text{ }^{\circ}\text{C}$. Both interaction pairs were measured 3 times over the 10 months and this data is presented below.

3.1.1 TrpA/TrpB Interaction Pair

As stated in Section 2.4.3 the ITC measurement parameters were adapted from the protocols used by Higara and Yutani [80].

3.1.1.1 ITC Measurements (Month = 0)

The first set of ITC measurements carried out on the TrpA/TrpB interaction pair, were in May of 2006 on a VP-ITC instrument at the UK MicroCal Headquarters in Milton Keynes.

The binding measurement was initially carried out at 25 °C rather than 40 °C, which was the temperature used by Hiraga and Yutani [80]. The majority of protein-protein binding assays are carried out at 25 °C and it was therefore logical to measure the binding parameters at this temperature. The heats produced at this temperature were relatively small, hence the experimental noise became significant and affected the base line as shown in Figure 3.1. In addition the binding sites were saturated very early in the experiment producing a steep binding transition, which was hard to accurately fit to a curve. Nevertheless, thermodynamic parameters were generated from the data and are displayed in Table 3.1. To try and increase the heats generated during the titration experiment, the temperature was set at 40 °C.

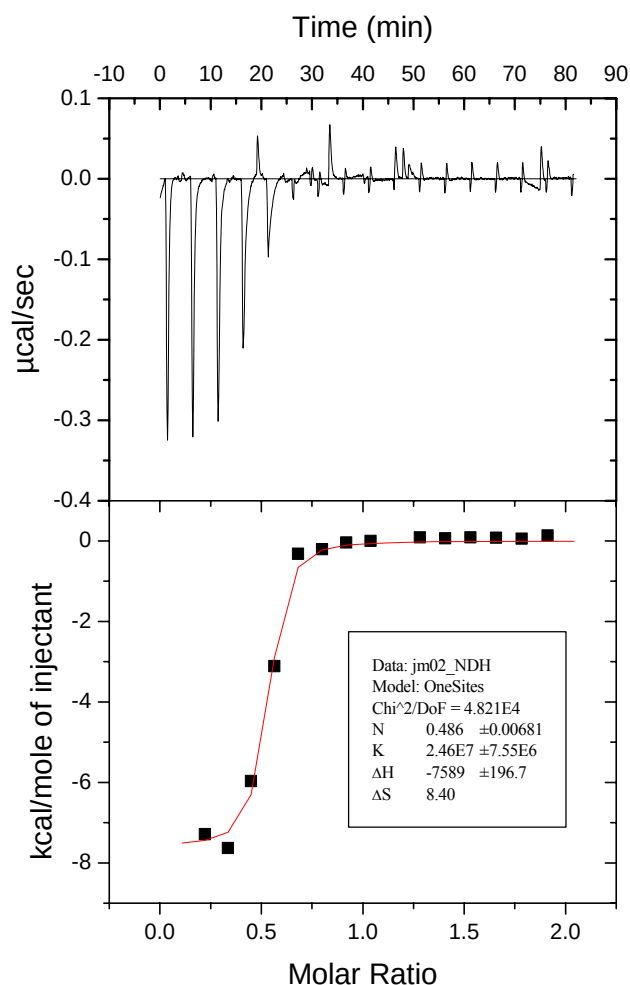


Figure 3.1: Interaction of TrpA and TrpB at 25 °C.

(Upper panel) The area under each peak represents heat released after each 15 μ l injection of 103 μ M TrpA into 10 μ M TrpB. (Lower panel) The binding isotherm obtained by plotting peak areas against the molar ratio of TrpA. The line represents the fitted curve obtained from nonlinear least squares regression analysis, assuming a one-site binding model.

The binding of TrpA with TrpB at 40 °C is shown in Figure 3.2. At this temperature the heats produced during the injections were larger ($\sim 2.5 \times$) than those seen for the 25 °C measurement, and the experimental noise contribution was greatly reduced. For this reason all other measurements involving the TrpA/TrpB interaction pair were carried out at 40 °C. Using these data points a binding isotherm was produced and a simple binding model fitted to it as described in Section 2.4.4. The curve generated through non-linear least squares regression analysis (NLLS), gave a binding affinity (K_A) of 1.96×10^7 which corresponded to a dissociation constant (K_D) of 5.10×10^{-8} Table 3.1.

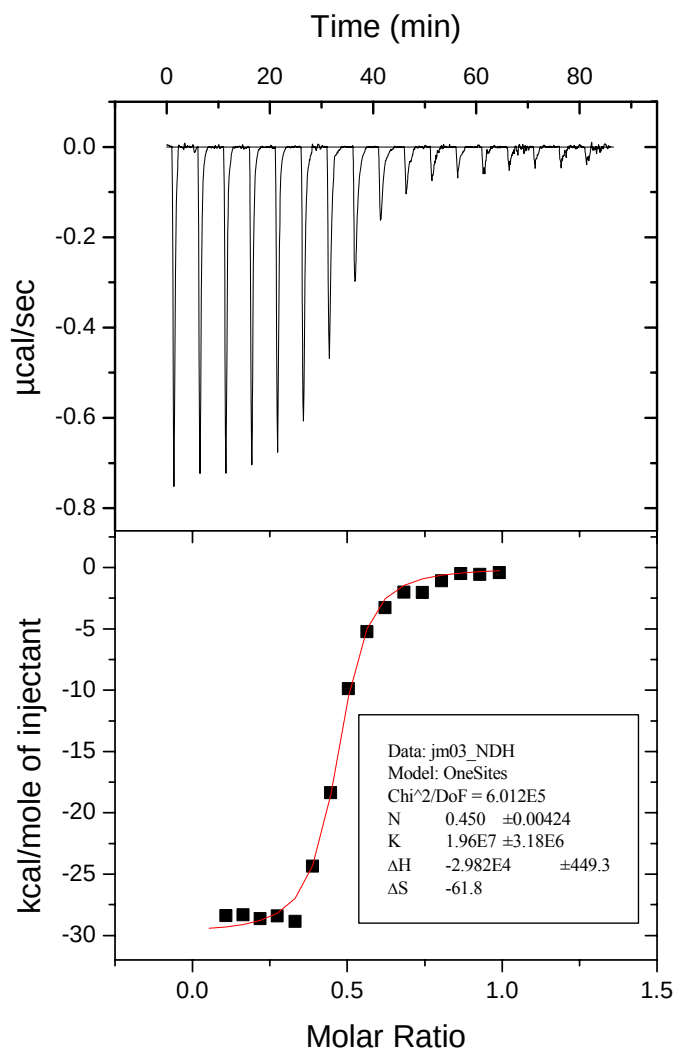


Figure 3.2: Interaction of TrpA and TrpB at 40 °C.

(Upper panel) The area under each peak represents heat released after each 15 μl injection of 50 μM TrpA into 10 μM TrpB. (Lower panel) The binding isotherm obtained by plotting peak areas against the molar ratio of TrpA. The line represents the fitted curve obtained from nonlinear least squares regression analysis, assuming a one-site binding model.

This value agreed well with the K_A of 1.11×10^7 reported by Higara and Yutani for the binding interaction in the presence of KCl [80]. In addition, the ΔH , $-\Delta S$ values were consistent with those reported in the literature, as shown in Table 3.1. The stoichiometry (N) of the reactions at both temperatures was around 0.5. This result was not consistent with that of Higara and Yutani who reported a stoichiometry of approximately 0.7 [80]. They blamed the deviation from the expected stoichiometry of 1:1 [101, 102] on incorporation of inactive β -subunit into the TrpB dimer. This explanation may be relevant here, but it seems unlikely that half of the TrpB would be produced in an inactive state, especially as the same purification protocols were followed as used by Higara and Yutani [80, 90]. The inconsistency in the stoichiometry values was not too serious as the thermodynamic parameters are

independent of the binding number and large errors in the stoichiometry are often observed, due, primarily, to over and under estimations of protein concentration¹

Comparison of the thermodynamic values obtained at 25 °C and 40 °C, revealed significant differences in the ΔH and $-T\Delta S$ parameters, as shown in Table 3.1. The K_A at the two temperatures was the same, within experimental error, but the ΔH at 25 °C was greatly reduced and the $-T\Delta S$ changed from being unfavourable at 40 °C to favourable at 25 °C. This kind of relationship in the variation of ΔH and ΔS , where much smaller overall ΔG changes, arise has been defined as entropy/enthalpy compensation [103-106]. Entropy/enthalpy compensation has been repeatedly observed for interaction systems that involve multiple, weak non-covalent bonds in an aqueous environment [103, 104]. It has been shown for entropy/enthalpy compensation resulting from variation of the temperature, that this phenomenon can be attributed to finite changes in heat capacity (ΔC_p) [107]. The ΔC_p for the interaction pairs was not investigated during this study.

	Temperature (°C)	K_A (M ⁻¹)	K_D (M)	ΔH (kJ·mol ⁻¹)	$-T\Delta S$ (kJ·mol ⁻¹)	N
Run1	25	$2.46 \pm 0.755 \times 10^7$	$4.07 \pm 1.28 \times 10^{-8}$	-31.8 ± 0.82	-10.5	0.486
Run2	40	$1.96 \pm 0.32 \times 10^7$	$5.10 \pm 0.83 \times 10^{-8}$	-125 ± 1.88	81.0	0.45
Higara and Yutani Values^a	40	1.11×10^7	9.05×10^{-8}	-119	76.6	0.7

Table 3.1: The thermodynamic parameters for the TrpA/TrpB interaction pair measured at 25 °C and 40 °C.

These thermodynamic parameters were measured in the buffer outlined in Section 2.3.7. ^a These values were taken from [80].

3.1.1.2 ITC Measurements (Month = 3)

The second set of measurements was carried out in July of 2006 on a VP-ITC instrument at the University of Sussex.

For these measurements the activity of the shoulder and main peak, observed in the gel filtration chromatogram of TrpB, were investigated. As described in Section 2.3.3 and shown in Figure 3.3, the main peak observed in the gel filtration step of the TrpB purification contained a shoulder. Analysis by SDS-PAGE found that both the shoulder and main peak contained a protein that ran as a single, identical bands corresponding to the molecular weight of TrpB (42.8 kDa). It was important to assess whether the activity of these two fractions was the same, or whether structural

¹ For further information regarding causes of these variations in the stoichiometry (N) please visit <http://www.chem.gla.ac.uk/staff/alanc/service1.htm>

differences, that are responsible for slightly different retention times on the gel filtration column, also changed the binding activity.

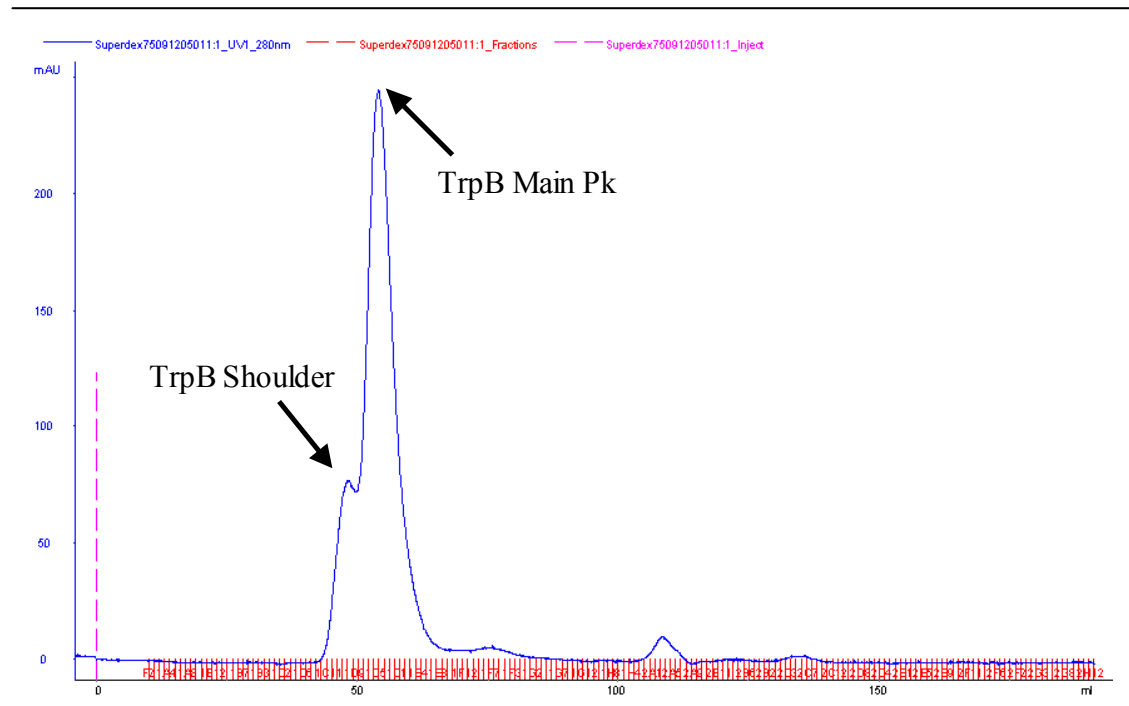


Figure 3.3: Superdex 75 16/60 column chromatogram for TrpB.

The main peak and shoulder corresponding to TrpB are indicated. A Superdex 75 16/60 column (GE Healthcare) equilibrated in 50 mM potassium phosphate pH 7.0, 300 mM KCl, 0.1 mM DTT, 5 mM EDTA, 0.02 mM PLP was used. This column was developed at 0.5 ml·min⁻¹ in the same buffer and the eluted fractions monitored at 280 nm.

ITC measurements were carried out at 40 °C, as before, and the resulting heats fitted to a one-site binding model. There was a difference in the appearance of the binding isotherms as shown in Figure 3.4. Even though the same concentrations of both protein components were used for both ITC runs, the binding transition for the TrpB shoulder fraction was far steeper than for the TrpB main peak fraction. The K_A for the shoulder fraction was approximately 63% of that for the main peak fraction (Table 3.2). All other thermodynamic parameters were similar within experimental error, however the stoichiometries were significantly different. Bearing in mind the dangers already highlighted in Section 3.1.1.1, in making interpretations based on differences in the binding number, it can be concluded that a large amount of the TrpB in the shoulder fraction was inactive. This was possibly due to mis-folding or non-covalent aggregation of the protein, which, while detected by gel filtration chromatography, could not be identified by SDS-PAGE.

In the context of producing quality interaction pairs for the purpose of inter-platform comparability applications, identifying differences in the purified product proteins was critical. Characterising the purification protocols to ensure they were robust and reproducible was part of this process. Data for the comparison of ITC and SPR was generated using the main peak fraction of TrpB.

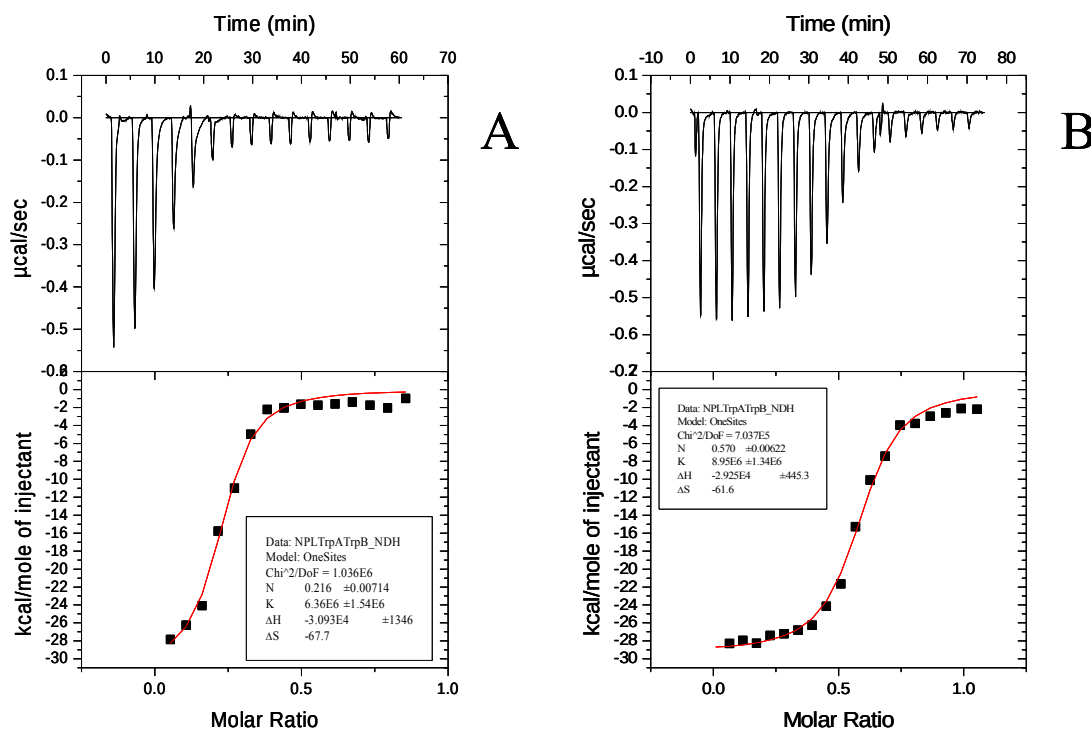


Figure 3.4: Interaction of TrpA with TrpB at 40 °C.

(A) Binding of TrpA with TrpB shoulder. (B) Binding of TrpA with TrpB main peak. (Upper panel) The area under each peak represents heat released after each 15 μ l injection of 50 μ M TrpA into 10 μ M TrpB. (Lower panel) The binding isotherm obtained by plotting peak areas against the molar ratio of TrpA. The line represents the fitted curve obtained from NLLS regression analysis, assuming a one-site binding model.

	K_A (M^{-1})	K_D (M)	ΔH ($kJ \cdot mol^{-1}$)	$-T\Delta S$ ($kJ \cdot mol^{-1}$)	N
TrpB Shoulder	$6.36 \pm 1.54 \times 10^6$	$1.57 \pm 0.39 \times 10^{-7}$	-129 ± 5.63	88.7	0.216
TrpB Main Peak^b	$1.01 \pm 0.17 \times 10^7$	$1.01 \pm 0.17 \times 10^{-7}$	-122 ± 1.86	80.3	0.575

Table 3.2: The thermodynamic parameters for the TrpA/TrpB interaction pair for the two TrpB gel filtration fractions.

These thermodynamic parameters were measured in the buffer outlined in Section 2.3.7. ^b The results shown here are the result of 4 independent measurements.

3.1.1.3 ITC Measurements (Month = 10)

As described in Section 2.5.2 all SPR measurements were carried out with 0.005% v/v of the surfactant P20 added to the binding buffers. P20 is added because the SRP instrument requires complex microfluidics to deliver material to the flow-cells and the presence of the surfactant reduces non-specific binding (NSB). In order to check

whether the presence of this detergent affected the binding energetics of the TrpA/TrpB interaction, ITC measurements were carried out in buffer with and without the addition of P20. Control experiments involving buffer-buffer injections and TrpA-buffer injections indicated that the presence of P20 did not change any heats of dilution (data not shown). With the exception of the addition of P20, the TrpA/TrpB interaction was measured under the same conditions as previously.

The observed binding isotherms for the TrpA/TrpB interaction in the absence and presence of P20 were very similar, as shown in Figure 3.5. The presence of the detergent appeared to have no effect on any of the binding parameters as shown by Table 3.3. This result was reassuring, as in order for the interaction pair to be useful as an inter-comparison material, it had to be functional on a number of different platforms. In their study, Hiraga and Yutani investigated the dominant types of inter-molecular interactions occurring between the subunits of Tryptophan Synthase and concluded there were large contributions from both hydrogen bonds, formed between specific residues, and electrostatic interactions [80]. They observed that increasing the concentration of salt in the buffer increased the K_A of the TrpA/TrpB complex. They suggested that this was due to the fact that there was electrostatic repulsion between the two subunits, which was diminished in the presence of the salt ions [80]. These observations indicate that hydrophobic forces do not play a dominant role in the TrpA/TrpB interaction and this may explain why the presence of P20 has no effect on the thermodynamic parameters of the interaction.

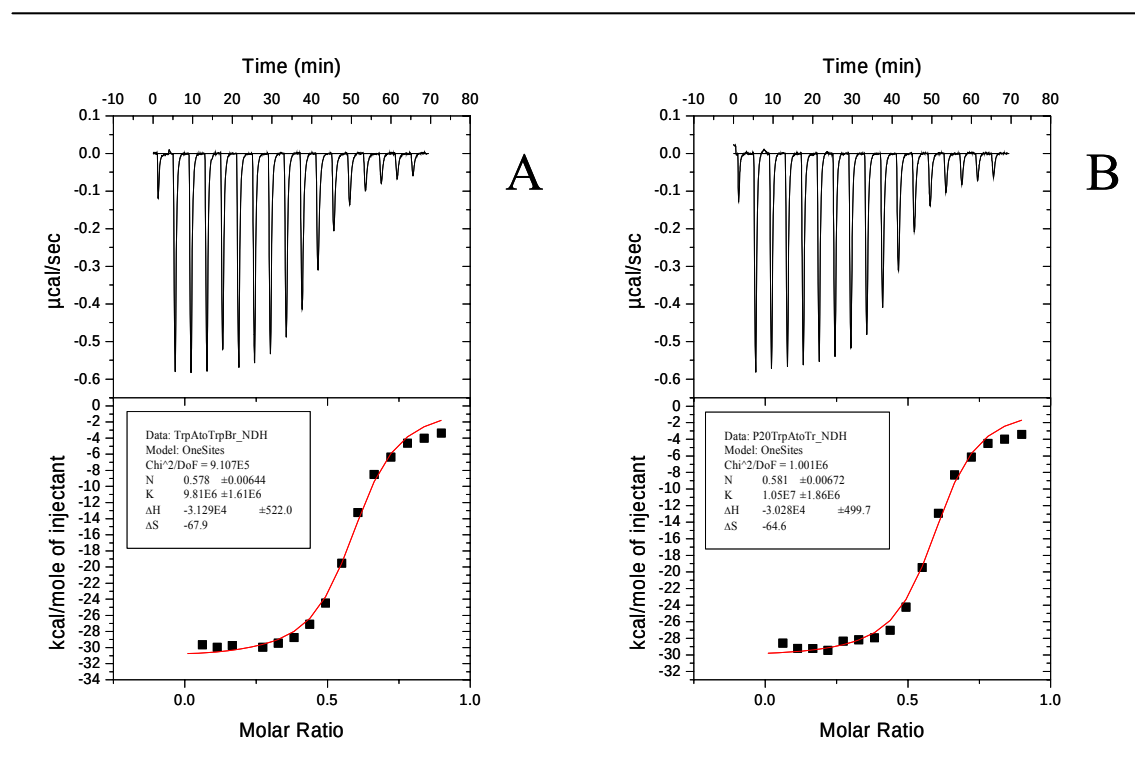


Figure 3.5: Interaction of TrpA with TrpB at 40 °C.

(A) Binding of TrpA with TrpB in the absence of 0.005% v/v P20. (B) Binding of TrpA with TrpB in the presence of 0.005% v/v P20. (Upper panel) The area under each peak represents heat released after each 15 μl injection of 50 μM TrpA into 10 μM TrpB. (Lower panel) The binding isotherm obtained by plotting peak areas against the molar ratio of TrpA. The line represents the fitted curve obtained from NLLS regression analysis, assuming a one-site binding model.

	K_A (M^{-1})	K_D (M)	ΔH ($kJ \cdot mol^{-1}$)	$-T\Delta S$ ($kJ \cdot mol^{-1}$)	N
– P20 ^c	$1.06 \pm 0.11 \times 10^7$	$9.48 \pm 1.01 \times 10^{-8}$	-129 ± 2.66	86.9	0.569
+ P20 ^c	$9.62 \pm 1.25 \times 10^6$	$1.05 \pm 0.14 \times 10^{-7}$	-129 ± 3.22	87.1	0.576

Table 3.3: The thermodynamic parameters for the TrpA/TrpB interaction pair in the presence and absence of 0.005% v/v P20.

These thermodynamic parameters were measured in the buffer outlined in Section 2.3.7. ^c The results shown here are the result of 2 independent measurements.

These results also indicated that the TrpA/TrpB interaction pair retained its binding activity over the 10-month storage period. This is discussed in more detail in Section 3.1.3.

3.1.2 NusA Cterm/LamN Peptide Interaction Pair

As stated in Section 2.4.3 the ITC measurement parameters were adapted from the protocols used by Bonin *et al* [84].

3.1.2.1 ITC Measurements (Month = 0)

The NusA Cterm/LamN peptide interaction pair was first studied using ITC at the same time as the initial TrpA/TrpB measurements were made at the MicroCal LLC UK Headquarters.

The measurement was carried out at 25 °C in order to replicate those measurements made by Bonin *et al* [84]. The results of the binding experiment are shown in Figure 3.6. The one-site binding model fitted the data very well and generated the thermodynamic parameters shown in Table 3.4. While the K_A reported by Bonin *et al* was the same within experimental error as the K_A for the NusA Cterm/LamN peptide interaction, there were notable differences in both the ΔH and $-T\Delta S$ [84]. These differences may well have been caused by the N-terminal biotin tag, which was added to the LamN peptide used in this study, but was not present in the peptide used by Bonin *et al* [84]. The biotin tag was added to the peptide to facilitate simple surface immobilisation, which is important for other surface-based interaction measurement platforms, as described in Section 1.4.2.4, but not required for ITC measurements. The biotin molecule was separated from the peptide by a 6-carbon spacer and it is quite possible that this spacer and biotin tag became conformationally “fixed” when the peptide bound to the protein due to the formation of non-specific, non-covalent bonds with some part of the protein surface. The loss of the rotational and translational entropy caused by this conformation “fixing” could well account for the unfavourable decrease in ΔS . The additional possible non-covalent contacts made by the biotin and linker tag, may account for the favourable decrease in ΔH .

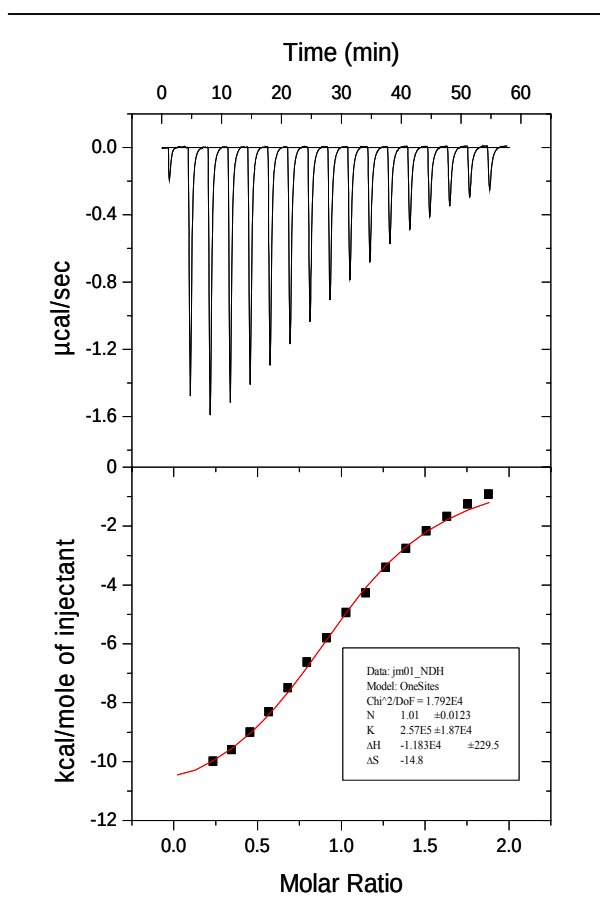


Figure 3.6: Interaction of NusA Cterm with LamN peptide at 25 °C (Month = 0).
 (Upper panel) The area under each peak represents heat released after each 15 μ l injection of 300 μ M LamN peptide into 30 μ M NusA Cterm. (Lower panel) The binding isotherm obtained by plotting peak areas against the molar ratio of LamN peptide. The line represents the fitted curve obtained from NLLS regression analysis, assuming a one-site binding model.

	K_A (M^{-1})	K_D (M)	ΔH ($kJ \cdot mol^{-1}$)	$-T\Delta S$ ($kJ \cdot mol^{-1}$)	N
NusA Cterm/LamN Peptide	$2.57 \pm 0.19 \times 10^5$	$3.89 \pm 0.28 \times 10^{-6}$	-49.5 ± 0.96	18.5	1.01
Bonin <i>et al</i> Values^d	$2.82 \pm 0.09 \times 10^5$	$3.57 \pm 0.11 \times 10^{-6}$	-39.5 ± 0.10	8.61	0.965

Table 3.4: The thermodynamic parameters for the NusA Cterm/LamN peptide interaction pair measured at 25 °C.

These thermodynamic parameters were measured in the buffer outlined in Section 2.3.7. ^d These values are taken from [84].

3.1.2.2 ITC Measurements (Month = 4)

Repeat measurements using the same interaction pair were carried out at 25 °C, 3 months later using a VP-ITC instrument at the University of Sussex. The binding data collected is shown in Figure 3.7 and Table 3.5.

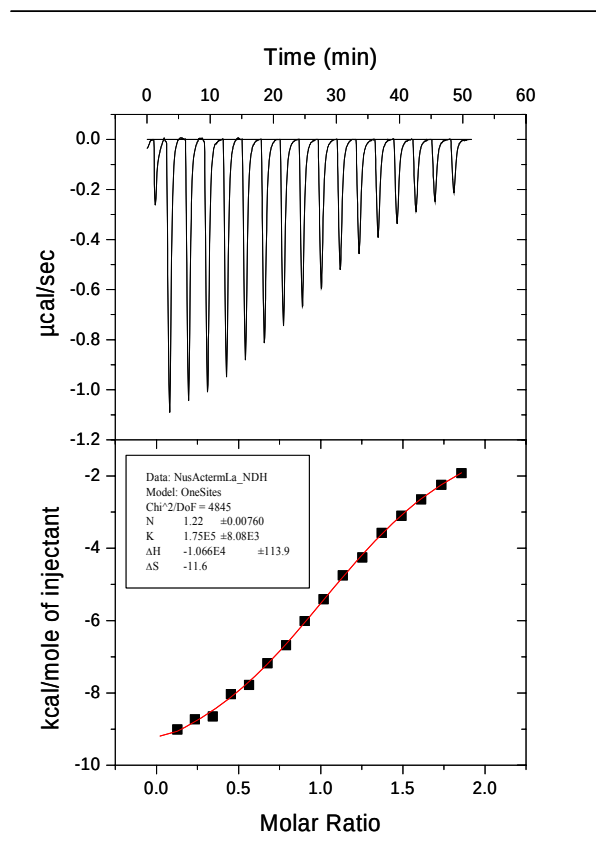


Figure 3.7: Interaction of NusA Cterm with LamN peptide at 25 °C (Month = 4).

(Upper panel) The area under each peak represents heat released after each 15 µl injection of 300 µM LamN peptide into 30 µM NusA Cterm. (Lower panel) The binding isotherms are obtained by plotting peak areas against the molar ratio of LamN peptide. The line represents the fitted curve obtained from NLLS regression analysis, assuming a one-site binding model.

The data indicated that the K_A of the interaction had dropped by 30% compared to the previous measurements shown in Table 3.4.

	K_A (M^{-1})	K_D (M)	ΔH ($kJ \cdot mol^{-1}$)	$-T\Delta S$ ($kJ \cdot mol^{-1}$)	N
+ 4 Months^e	$1.81 \pm 0.15 \times 10^5$	$5.56 \pm 0.50 \times 10^{-6}$	-43.6 ± 0.97	13.5 ± 1.08	1.19

Table 3.5: The thermodynamic parameters for the NusA Cterm/LamN peptide interaction pair measured at 25 °C after 4 months of storage at –80 °C.

These thermodynamic parameters were measured in the buffer outlined in Section 2.3.7. ^e The values shown here are an average of 3 independent measurements.

3.1.2.3 ITC Measurements (Month = 10)

Further measurements using the NusA Cterm/LamN peptide were subsequently performed after proteins had been stored for 10 months at -80°C . A VP-ITC instrument at NPL was used. The measurements were carried out under the same conditions outlined for the previous measurements (Sections 3.1.2.1 and 3.1.2.2). The binding isotherm and curve fitting is shown in Figure 3.8 and the calculated thermodynamic parameters are listed in Table 3.6.

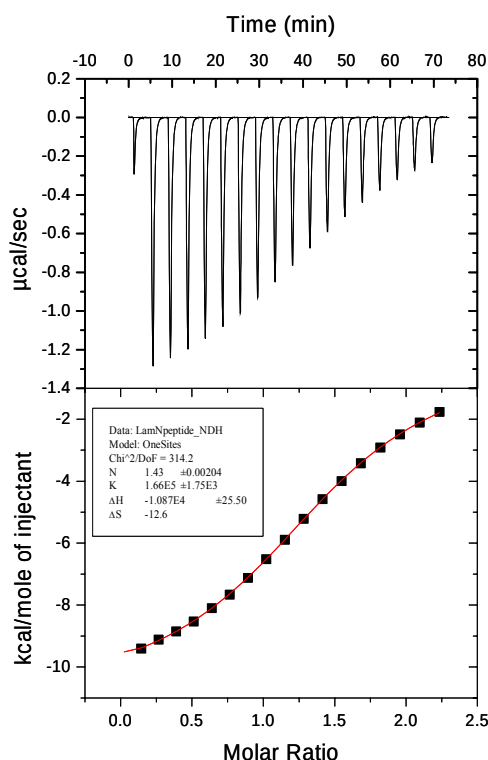


Figure 3.8: Interaction of NusA Cterm with LamN peptide at 25 °C (Month = 10).

(Upper panel) The area under each peak represents heat released after each 15 μl injection of 350 μM LamN peptide into 30 μM NusA Cterm. (Lower panel) The binding isotherms are obtained by plotting peak areas against the molar ratio of LamN peptide. The line represents the fitted curve obtained from NLLS regression analysis, assuming a one-site binding model.

The data again indicated that the interaction pair binding activity had decreased during storage, this time by 39% from the initial binding measurements made in May 2006 (Table 3.4). Both these and the results presented in Section 3.1.2.2 are discussed in greater depth in Section 3.1.3.

	K_A (M^{-1})	K_D (M)	ΔH ($kJ \cdot mol^{-1}$)	$-T\Delta S$ ($kJ \cdot mol^{-1}$)	N
+ 10 Months ^f	$1.58 \pm 0.03 \times 10^5$	$6.35 \pm 0.48 \times 10^{-6}$	-45.5 ± 0.62	15.8 ± 0.18	1.60

Table 3.6: The thermodynamic parameters for the NusA Cterm/LamN peptide interaction pair measured at 25 °C after 10 months of storage at –80 °C.

These thermodynamic parameters were measured in the buffer outlined in Section 2.3.7. ^f The values shown here are an average of 3 independent measurements.

3.1.3 Stability of the Interaction pairs

3.1.3.1 TrpA/TrpB Interaction Pair

By carrying out multiple binding measurements over an extended period of time (10 months) it was possible to assess the stability of both interaction pairs in terms of their binding activity. For these proteins to be useful as inter-comparison materials it was critical that they retained their activity during storage for an extended period of time.

The stability of the binding activity of the TrpA/TrpB pair over the 10-month time frame was excellent, as shown in Figure 3.9. The ΔG values, which correspond to the K_A , were very reproducible, while the other thermodynamic parameters were also consistent. Both components of the interaction pair were stored at –80 °C between measurements, and the results indicated that the freeze-thaw process appeared to have no adverse effect on the activity of either protein.

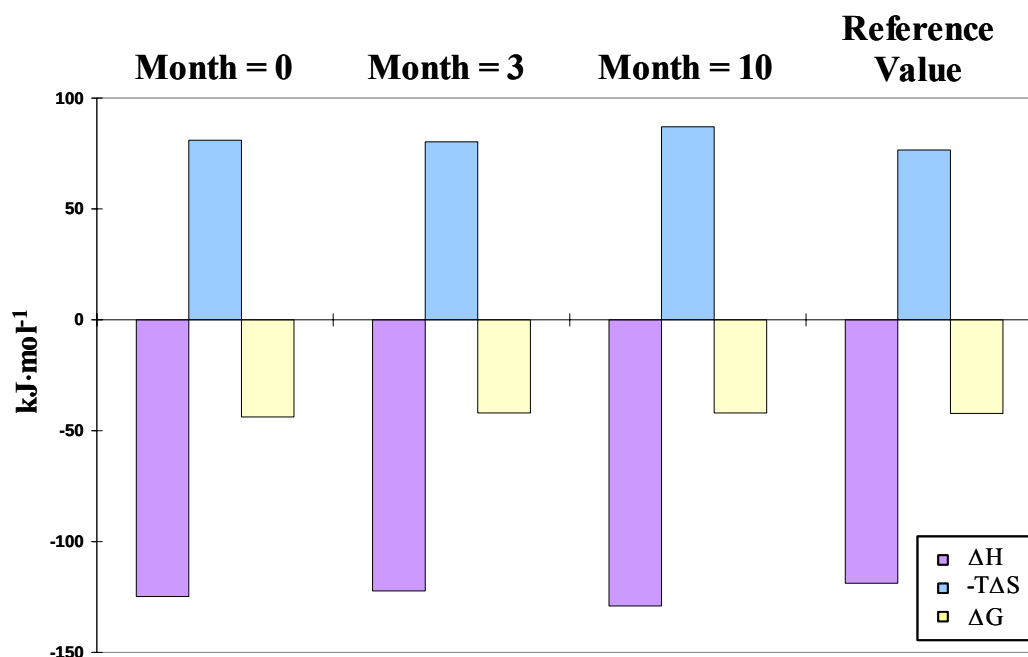


Figure 3.9: Comparison of the main thermodynamic parameters for the TrpA/TrpB interaction pair measured over a period of 10 months.

ΔG was calculated from the K_A using Equation 2.2. All data shown here were measured at 40 °C in the TrpA/TrpB binding buffer listed in Section 2.3.7. The data shown for the reference value was taken from [80].

3.1.3.2 NusA Cterm/LamN Peptide

Similar binding measurements made over time using the NusA Cterm/LamN peptide interaction pair (Figure 3.10) showed that this pair appeared to lose some binding activity with time in storage – as the ΔG values increased from $-31.0 \text{ kJ}\cdot\text{mol}^{-1}$ to $-29.7 \text{ kJ}\cdot\text{mol}^{-1}$, corresponding to 39% decrease in binding affinity. This decrease was significant with respect to the experimental errors. In addition the stoichiometry of the interaction increased from $N = 1.01$, (the expected value), to $N = 1.60$ after 10 months of storage. An increase in the binding number indicates that a greater quantity of the ligand is required to occupy half of the binding sites. As discussed in Section 3.1.1.1 interpretation of the binding number is often difficult due to errors associated with protein concentration estimation. In this case however, protein concentrations were measured before the first measurement (Month = 0) and then identical stored samples were used for all other measurements. This meant that errors, due to protein concentration estimation played no part. The change in the stoichiometry must have been due to a change in one of the binding components. It appears from the data that the peptide rather than the protein lost some activity. If it were the protein, then the stoichiometry would have decreased. While degradation of the peptide cannot be ruled out, it is unlikely, and the apparent decrease in active peptide may be due to non-specific adsorption to the tube wall.

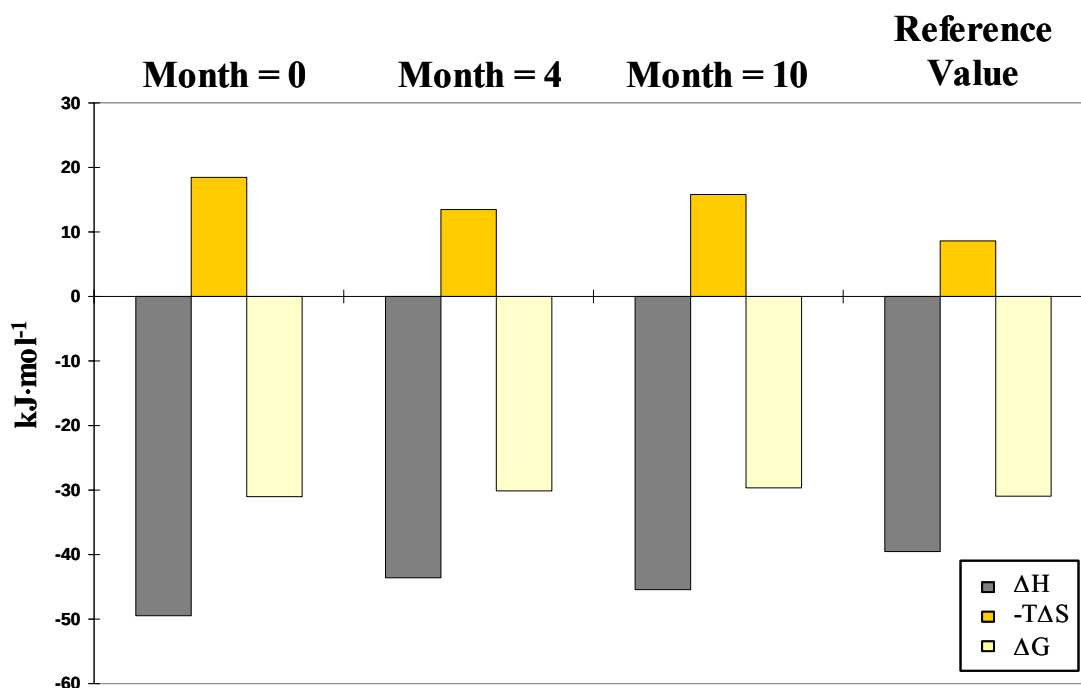


Figure 3.10: Comparison of the main thermodynamic parameters for the NusA Cterm/LamN peptide interaction pair measured over a period of 10 months.

ΔG was calculated from the K_A using Equation 2.2. All data shown here were measured at 25°C in the NusA Cterm/LamN peptide binding buffer listed in Section 2.3.7. The data shown for the reference value were taken from [84].

3.2 Surface Plasmon Resonance (SPR)

3.2.1 TrpA/TrpB Interaction Pair

Immobilisation of protein to a surface can alter the mode and kinetics of the interaction with its binding partner. To study this we therefore sought to prepare surfaces where both of the interaction partners were individually immobilised i.e. were ligands in the binding reaction. The list of possible immobilisation strategies commonly used with the Biacore platform and those used in this study with an explanation are shown in Table 3.7.

Immobilisation Strategy	Ligand	Notes
Amine Coupling	TrpA	Generated active surface (Section 3.2.1.3)
	TrpB	Produced inactive surface (Section 3.2.1.1)
Thiol Coupling (maleimide)	TrpA	Produced inactive surface (Section 3.2.1.5)
	TrpB	Produced inactive surface (Section 3.2.1.2)
Carboxyl Coupling	TrpA/TrpB	Both TrpA and TrpB contain acidic bases critical for binding – not attempted
Antibody Directed	TrpA/TrpB	No suitable TrpA, or TrpB antibody commercially available – not attempted
Linker (Biotin)	TrpA/TrpB	Labelling could alter binding kinetics – independent of platform – not attempted

Table 3.7: Immobilisation strategies for the TrpA/TrpB interaction pair.

3.2.1.1 TrpB Ligand: Amine Coupling

TrpB exists as a homodimer in solution [75]. To avoid complex, bivalent binding kinetics at the surface the first approach attempted was to immobilise TrpB. It was expected that the immobilisation of TrpB would produce a heterogeneous surface with a mixture of TrpB dimers (where both TrpB molecules were attached to the surface) and monomer units covalently attached to the surface.

Injection of 10 μ M TrpA to the chip surface displayed low amounts of non-specific binding (NSB) (typically 1-3 RU) therefore the CM5 chip was deemed suitable for the proposed binding study. TrpA has a calculated pI of ~ 5.7 [108], pre-concentration suggested that the optimum buffer to use was 10 mM sodium acetate pH 5.0, or 5.5 (data not shown). Immobilisation of TrpB at pH 5.5 produced a total increase of 405 RU, however when 200 nM of TrpA was injected over the surface for 120 seconds no significant binding was observed (data not shown). It was therefore assumed that amine directed immobilisation had either inactivated the TrpB, or blocked free access to its binding site. Repeating the amine coupling on a fresh chip at

pH 5.0 assessed the effect of immobilisation pH. Similar amounts of TrpB were bound to the chip, however no binding activity was observed (data not shown).

3.2.1.2 TrpB Ligand: Thiol Coupling

Amine coupling had produced an inactive surface; therefore thiol coupling was attempted to produce a more ordered orientation for TrpB. The TrpB homodimer is reported to contain five cysteine residues in the denatured state as determined by the Ellman's assay [109]. However in the native state at pH 7.6 only a single cysteine (per monomer) reacts with Ellman's reagent, previously this has been exploited to site specifically label the protein with a fluorescent dye [102]. The enzymatic activity of the resulting labelled TrpB was ~ 41% that of the native protein, dependent on the nature of the label [102]. Though TrpB enzymatic activity does not directly relate to TrpA/TrpB binding affinity, formation of the complex does result in an increase in the turnover of both reactions [101, 102, 110]. This cooperativity between the two subunits implies that any change in complex stability will result in decreased catalytic activity and vice versa.

It was probable that thiol coupling TrpB to a Biacore chip would impair, but not abolish, the binding to its interaction partner TrpA. The highest rates of immobilisation were achieved at pH 4.5, with ~ 270 RU of TrpB being bound to the chip after ethanolamine blocking. When 200 nM TrpA was injected over the surface for 120 seconds a small amount of binding ~ 2 RU was observed, however this was attributed to NSB which was observed in another flow-cell that contained bound protein (NusA Cterm). It was concluded that immobilisation of TrpB via either amine, or thiol coupling appeared to inhibit binding to its interaction partner, suggesting that TrpB has limited use as a standard where its covalent attachment to a solid phase is required.

3.2.1.3 TrpA Ligand: Amine Coupling

Due to the lack of binding activity displayed by immobilised TrpB (Sections 3.2.1.1 and 3.2.1.2) TrpA immobilisation to the CM5 chip was attempted. It was possible that the kinetics from TrpA immobilisation could be complicated due to the TrpB homodimer analyte binding in a bivalent manner, depending on the density of the ligand. Heterogeneous binding at the surface might occur (a mix of 1-to-1 and bivalent) which would prove hard to model. To determine the effect of a heterogeneous surface on the overall binding kinetics TrpA was immobilised at various densities on the surface in multiple flow-cells.

To prepare a suitable TrpA surface for kinetic experiments, optimisation of the running buffer and immobilisation conditions needed to be performed. The TrpA/TrpB running buffer was supplemented with 0.005% v/v P20 to remove the small amount (< 5 RU) of NSB observed in Fc1 when using the buffer without surfactant. Pre-concentration experiments to determine optimum immobilisation conditions indicated that 10 mM sodium acetate pH 4.5 yielded the highest level of binding to the CM5 chip. To reduce mass transport limitations for the surface Equation 2.4 was used to estimate the required amount of ligand that needed to be immobilised so that $R_{\max}$ was in the range for ideal kinetic experiments (100-200 RU). Fc3 was immobilised with 150 RU of TrpA, however the surface test, where the analyte was flowed over the immobilised ligand, only generated an $R_{\max}$ of ~ 117.5 RU. Depending on the stoichiometry of the interaction, the activity of the

surface was less than 50% suggesting this was not an ideal model to examine TrpA/TrpB binding kinetics. However, given that other models/immobilisation procedures displayed no activity, or were not suitable, full kinetic analysis was performed using the amine coupled TrpA model and is reported here.

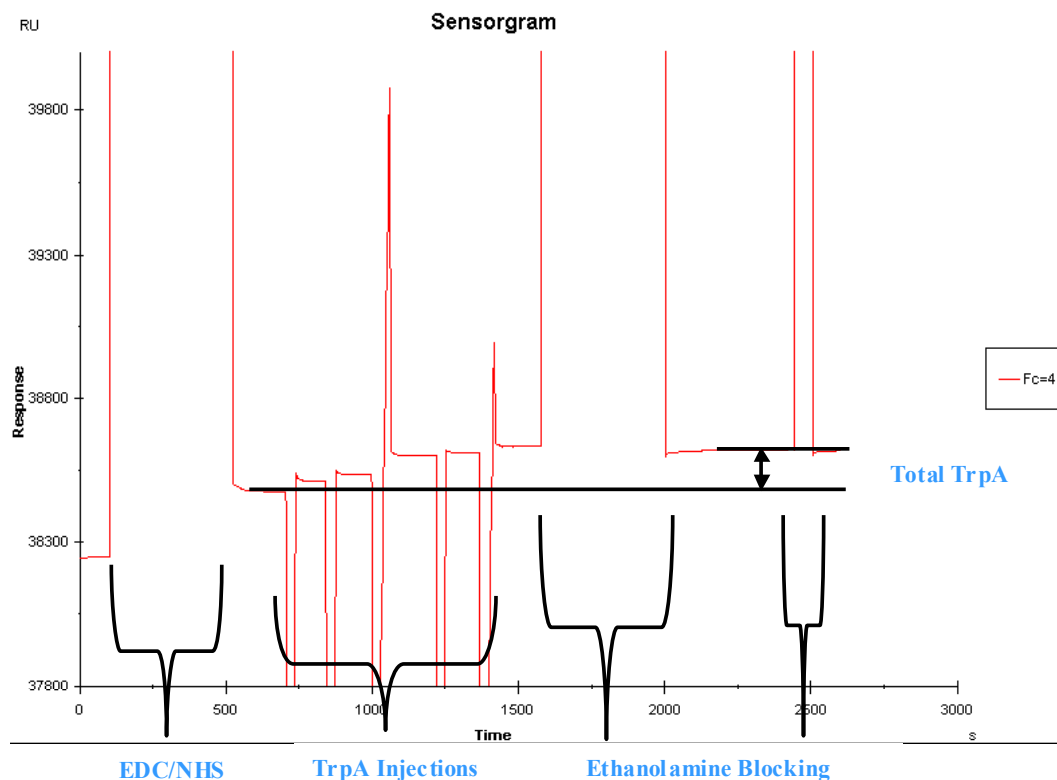


Figure 3.11: Sensorgram showing TrpA immobilisation to a CM5 chip. Each of the phases of the immobilisation cycle is highlighted on the sensorgram. Total TrpA represents the total amount ~ 140 RU of ligand covalently attached to the CM5 chip.

Regeneration of the TrpA surface after a binding cycle was required due to the incomplete dissociation of the analyte after 10 minutes. Regeneration scouting allows the optimum conditions to be determined in terms of complete regeneration, stable baseline and retention of the ligand activity on the surface. Previously, washing for 30 seconds with 10 mM NaOH resulted in complete loss of activity; therefore regeneration scouting was performed with NaOH omitted. The optimum regeneration conditions in terms of baseline stability, ‘complete regeneration’ and retention of binding activity was 4 M MgCl₂ (data not shown). All regeneration phases were performed using a 60 seconds injection of 4 M MgCl₂ followed by a 240 seconds stabilisation period where running buffer was flowed over the chip to allow baseline stabilisation before commencing the next cycle. The performance of the chip and type of interaction was then assessed before carrying out kinetic analysis.

Surface performance was assessed by carrying out repeat binding/regeneration cycles using 100 nM of TrpB. Baseline stability was ± 1 RU and steady-state binding generated average binding levels of 78 ± 0.5 RU, over 15 repeat cycles, using 3 start-up runs to normalise the system. These values were considered suitable for use in kinetic analysis.

Linked reactions describe a system where, in a time-dependent manner, binding of the analyte is followed by a conformational change in the analyte-ligand complex, or a second binding event, resulting in a stabilisation of the complex and a change in the dissociation rate. Linked reactions can be identified by looking at the relationship between association time and the rate of dissociation. Since the conformational change or second binding event is time dependent, longer association times will result in a higher degree of complex stabilisation on the surface and therefore slower dissociation rates. Figure 3.12 shows that the rate of dissociation was time dependent as the dissociation curves are similar for analyte injections of 30, 180 and 600 seconds. A second immobilisation onto a new chip also displayed a time-dependent dissociation curve (data not shown). This data suggested that a more complex model other than 1-to-1 binding was occurring at the surface of the sensor, therefore the kinetic data was fitted to various models to determine the type of interaction.

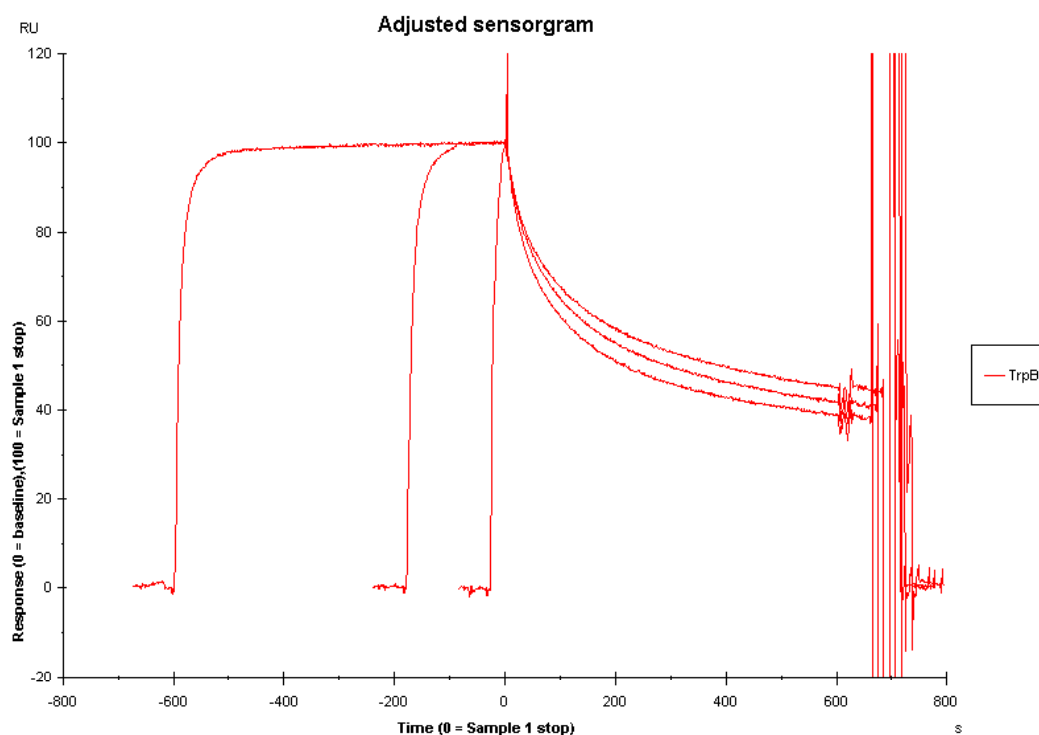


Figure 3.12: Control for Linked Reactions in Fc4.

Injections of 2500 nM TrpB at various lengths (30, 180 and 600 seconds) were analysed at a flow rate of $10 \mu\text{l}\cdot\text{min}^{-1}$ for changes in the shape of the dissociation curve. The sensorgrams have been adjusted so the association curves were normalised to 100, allowing comparison of sensorgrams that have not reached steady state.

Mass transport limitation occurs when either the rate of association, or disassociation, contains a significant component due to the rate of transport of the analyte to and from the chip surface. Lowering the density of immobilised ligand, or increasing the flow rate can reduce mass transport limitations. The binding and dissociation rates at various flow rates (10 , 30 and $75 \mu\text{l}\cdot\text{min}^{-1}$) were compared. The observed binding and dissociation rates were the same for Fc3 (data not shown) and Fc4 for all three flow

rates as shown in Figure 3.13, this suggested that the surface was not mass transport limited.

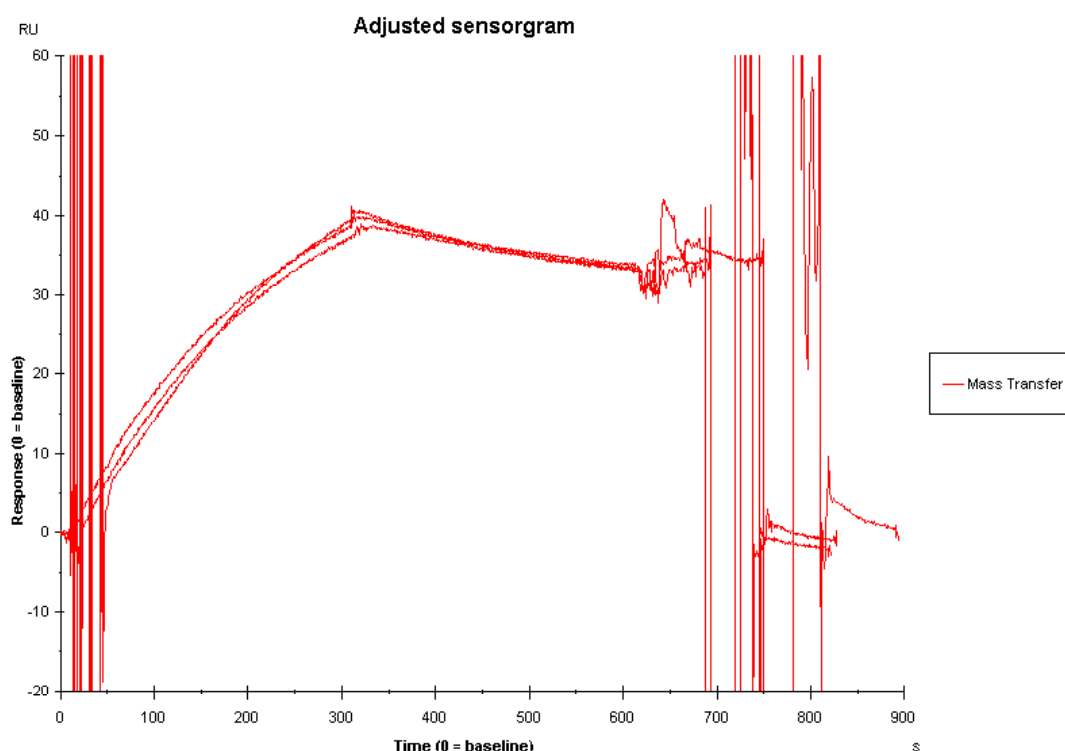


Figure 3.13: Control for Mass Transfer in Fc4.

The association rates for the injection of 100 nM TrpB at the flow rates of 10, 15 and 75 $\mu\text{L}\cdot\text{min}^{-1}$ were analysed to look for mass transport limitations. The sensorgrams have been adjusted to zero response and time at the baseline report point.

The performance and mass transport controls suggested that no mass transport contributions were present and that the model was suitable to use in kinetic analysis. For the kinetic analysis, the concentration of the analyte (TrpB) was expressed as a monomer, rather than the dimer. An example of the binding curves and associated fitting data is shown in Figure 3.14. The reduced χ^2 value and the residuals of the curve fit were used to assess how well the proposed model fitted to the data (Table 3.8) and (Figure 3.14). Sharp spikes on the sensorgram around the sample injection and sample stop points were due to poor reference subtraction for Fc4 – Fc1. Ideally, when using Fc4 as the active surface, Fc3 would generate a better reference subtraction than Fc1. Fc3 was not used as a blank as it was deemed more important to have at least two active flow-cells in order to analyse any effects that immobilisation density might play on the type of interaction on the chip surface.

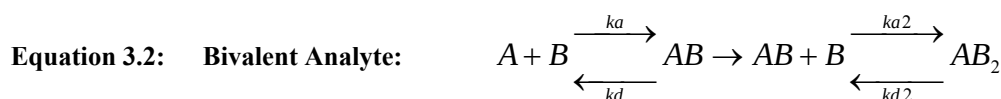
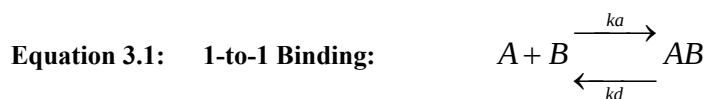
The kinetic data from both Fc3 and Fc4 can be modelled by either 1-to-1 (Equation 3.1), or bivalent analyte (Equation 3.2) models as shown in Figure 3.14. Ideally the modelling of a kinetic response will display a low reduced χ^2 value (≤ 2) and residuals that display a random distribution (homoscedastic) and ideally less than 5 RU spread. Examination of the reduced χ^2 values and residuals indicated that the bivalent model was justified over the 1-to-1 model. This and the results of the linked reaction control suggested a time dependent second binding interaction was occurring.

The ITC data and literature [75] suggest that TrpB forms a homodimer in solution. Therefore this evidence and the Biacore data indicate a bivalent model. A 2-state model could be supported by the linked-reaction control; however there is a poor fitting to this model (reduced $\chi^2 > 5$) and no other evidence to support a conformational change upon binding suggests this model is not valid.

For the bivalent model the surface activity of TrpA was estimated at 50% for Fc3 and 48% for Fc4 using Equation 2.4 (assuming a binding stoichiometry of 1). The low activity, indicated that the majority of the TrpA on the surface was either inactive, or in the incorrect orientation to bind TrpB.

Estimated dissociation constant:-

Using a bivalent model the average K_D for the first site interaction (K_{D1}) was 70 ± 4.3 nM using data measured at 40 °C from Fc 3 and 4 (Table 3.8). The second site interaction (avidity) did not produce a SPR response as the analyte was already in close proximity to the surface. The association rate constant for this interaction was therefore reported in units of $\text{RU}^{-1} \cdot \text{s}^{-1}$. However, this value can be converted to $\text{M}^{-1} \cdot \text{s}^{-1}$ using Equation 3.3, allowing the overall affinity K_D to be determined for the second site interaction. The average K_D of this second site was 430 ± 56.6 nM at 40 °C again using data from Fc3 and Fc4. The dissociation constant for the second interaction was higher than that for the first, however this could be explained as the interaction might be constrained by the first site. The larger error on the second site interaction probably represented the uncertainty in estimating the affinity and the conversion factor used to generate the association constant.



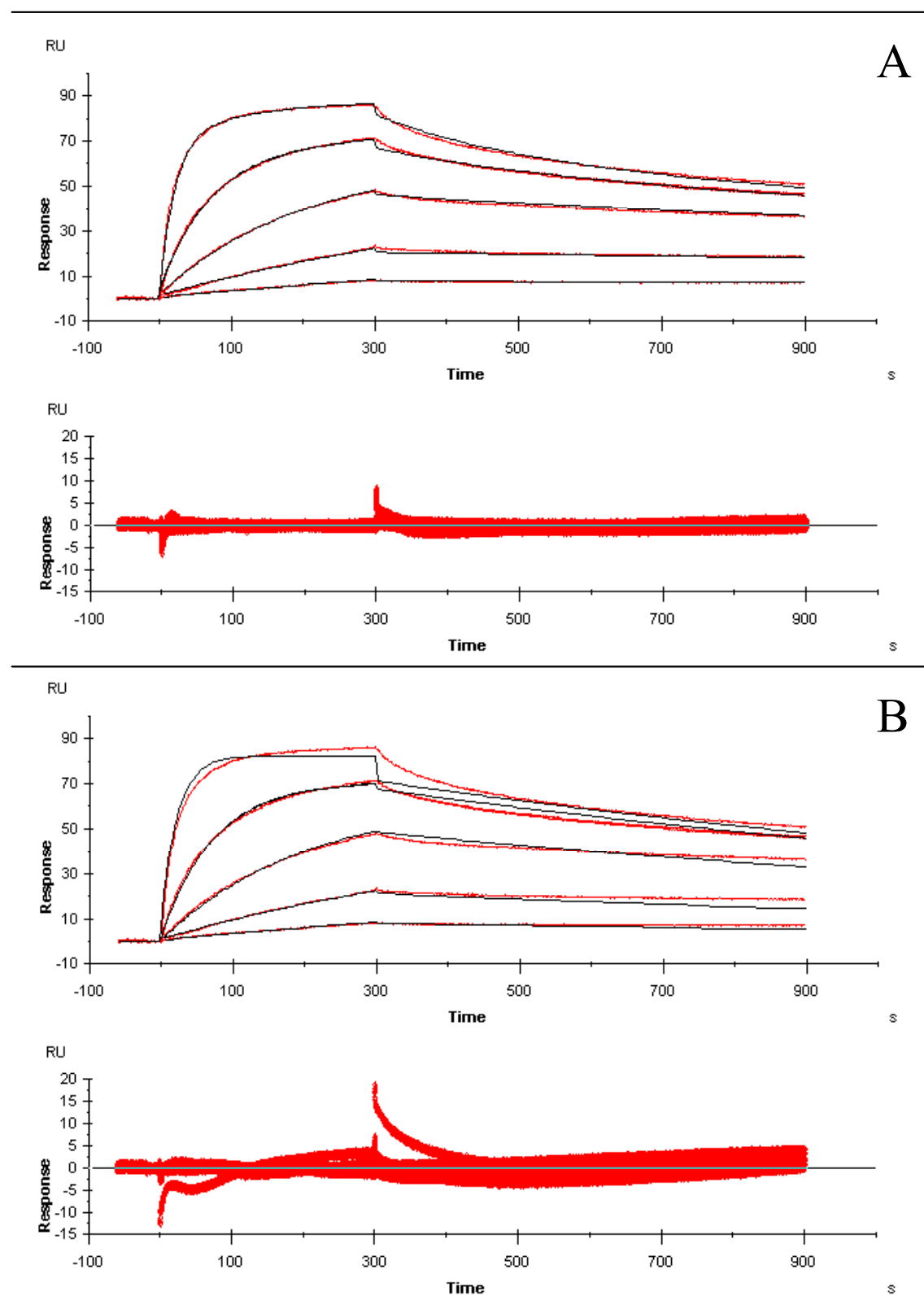


Figure 3.14: Model-fitting to the TrpA-TrpB kinetic data.

(A) Sensograms (upper panel) and residual plots (lower panel) represent the TrpA/TrpB kinetic data fitted to a Bivalent model. (B) Sensograms (upper panel) and residual plots (lower panel) fitted to a 1-to-1 model. Kinetic reactions were performed using TrpB concentration from 1-333 nM. Samples were injected for 300 seconds and dissociation was monitored for 600 seconds. Regeneration was performed using a 60 seconds injection of 4 M MgCl_2 and the chip was allowed to stabilise for 240 seconds before the next binding cycle.

	Fc4 (R_{max} 67 RU)	T value	Fc3 (R_{max} 117 RU)	T value
ka1 (1·M⁻¹·s⁻¹)	36880	580	36050	390
kd1 (1·s⁻¹)	0.002465	330	0.002629	140
ka2 (1·RU⁻¹)	0.001234	100	0.000858	46
kd2 (1·s⁻¹)	0.00412	120	0.003454	51
R_{max} (RU)	71.7	1100	121.5	590
Chi²	0.284	-	0.438	-
K_D1	6.68×10^{-8}	-	7.29×10^{-8}	-
^ska2 (1·M⁻¹·s⁻¹)	1.06×10^4	-	7.36×10^3	-
K_D2	3.90×10^{-7}	-	4.70×10^{-7}	-

Table 3.8: Kinetic parameters for TrpA/TrpB interaction from Fc 3 and 4 demonstrating the repeatability of binding.

Data was fitted to a bivalent model, with ^ska2 converted from 1·RU⁻¹ to 1·M⁻¹·s⁻¹ using Equation 3.3. The T-value represents the standard error and was obtained by dividing the parameter by the standard error of that parameter. T-values greater than 10 were considered significant, good quality data will display T-values > 100.

Equation 3.3: $k_{a2} \cdot MW \cdot 100$

(Bivalent kinetics second site converting RU⁻¹·s⁻¹ to M⁻¹·s⁻¹: CM5 chip only)

For the Biacore T100 the recommended amount of surfactant used to supplement running buffers is 0.05% v/v P20. Initially we sought to match the buffers from the ITC experiments and omitted P20, however, it was found that omission of surfactant increased the amount of NSB and produced poor quality data. Kinetic runs were repeated with running buffer supplemented with 0.05% v/v P20 with no significant change observed in the TrpA/TrpB interaction kinetics.

Storage of the chip in TrpA/TrpB assay buffer at 4 °C for prolonged periods resulted in a loss of binding for the chip as observed by the decreasing R_{max} value for the surface (~ 25% loss after each storage period). This suggested that there was limited utility of the TrpA/TrpB interaction pair for solid phase affinity standards if chips were to be stored outside of the instrument for long periods.

3.2.1.4 TrpA Ligand: Thermodynamics

Thermodynamics were performed on the Biacore for the TrpA/TrpB pair to obtain more information about the interaction. The thermodynamic data could be directly compared to that determined using ITC to check whether immobilisation had altered the TrpA/TrpB interaction. The thermodynamics of the interaction was assessed by performing kinetic analysis at a range of temperatures from 4-40 °C. Fitting the resulting kinetic data to both 1-to-1 and bivalent (binding site 1) interaction models produced a complex response that was not sufficiently modelled by the thermodynamic analysis program supplied by Biacore (data not shown). The association rate displayed a linear response with increasing temperature, however the dissociation rate exhibited a 'complex' biphasic response, which suggested another process could be altering the kinetics. Therefore these results could not be compared with those of the ITC.

3.2.1.5 TrpA Ligand: Thiol Coupling

To confirm the kinetic data obtained with amine coupling in Section 3.2.1.3 another immobilisation strategy was attempted. TrpA contains 3 cysteine residues that are reported to be surface exposed, however only a single cysteine was reported to react with the bulky Ellman's reagent in its native state [102]. In addition fluorescent labelling of TrpA was shown not to significantly effect the association of TrpA with the TrpB dimer [102]. Hence it was assumed that immobilisation using thiol coupling might generate an active surface.

Immobilisation to the thiol-modified surface (Section 2.5.4) at pH 4.5, generated a TrpA surface of ~ 200 RU. When 200 nM TrpB was injected over the surface no binding was observed, suggesting the TrpA had been inactivated, or there was restricted access to the binding site. No other direct immobilisation strategies were deemed suitable for TrpA attachment to the chip, therefore only the amine-directed data could be used in the overall analysis of the TrpA/TrpB interaction.

3.2.2 NusA Cterm/LamN Peptide Interaction Pair

Table 3.9 lists the possible immobilisation strategies for the NusA Cterm/LamN peptide pair. The LamN peptide was N-terminally biotinylated allowing additional immobilisation approaches through this tag. The calculated pI of NusA Cterm is 3.91. This did not suit the standard amine coupling methods due to the limitation of pre-concentration on the standard carboxylated CM chips.

Immobilisation Strategy	Ligand	Notes
Amine Coupling	NusA Cterm	Potential problem due to pI (Section 3.2.2.1)
	LamN Peptide	Lysines required for protein binding: not attempted
Carboxyl Coupling	NusA Cterm	Carboxylates required for protein binding: not attempted
	LamN Peptide	No carboxylate group on peptide: not attempted
Thiol Coupling	NusA Cterm	Section 3.2.2.2
	LamN Peptide	No thiol groups in peptide: not attempted
Antibody Directed	NusA Cterm	No suitable commercial antibody: not attempted
	LamN peptide	Anti-biotin could be used: not attempted
Linker (Biotin)	LamN Peptide	Section 3.2.2.3

Table 3.9: Immobilisation strategies for NusA Cterm/LamN peptide interaction pair.

3.2.2.1 NusA Cterm Ligand: Amine Coupling

Direct amine coupling of NusA to a CM5 chip was attempted, even though, due to the low pI of the protein, the system would require optimisation to be successful. The NusA Cterm stock was stored in NusA Cterm/LamN peptide binding buffer (Section 2.3.7), which used Tris as the buffering agent. Tris is incompatible with both amine and thiol coupling methods due to the presence of secondary amines, therefore before immobilisation NusA Cterm was buffer exchanged into phosphate buffered saline (PBS) from Sigma-Aldrich (10 mM phosphate buffer, 2.7 mM KCl, 137 mM NaCl, pH 7.4). The estimated pI of NusA Cterm was not compatible with pre-immobilisation, as at buffers $\geq$ pH 4.0 the charge of the protein repelled rather than attracted it to the dextran surface of the chip. The CM5 chips pKa is approximately 4.0, therefore lowering the pH of the immobilisation buffer below pH 4.0 would have generated positively charged dextran and protein, again resulting in protein-surface

repulsion. The protein can be chemically modified to neutralise the carboxyl functional groups increasing the pI of the protein, however for NusA Cterm this was not deemed suitable, as many of the acidic amino acids are vital for LamN peptide binding [84].

Pre-concentration of 20 $\mu\text{g}\cdot\text{ml}^{-1}$ NusA Cterm was attempted using sodium acetate buffer prepared at pH, 3.5, 3.6, 3.7, 3.8, 3.9, 4.0, 4.5, 5 and 5.5 and HBS-N at pH 7.0. Pre-concentration only occurred at pH 3.5 and 3.6, therefore immobilisation was attempted in these buffers. While pre-concentration occurred at this pH, no protein remained on the chip when HBS-EP was passed over the surface. This indicated that the rate of EDC/NHS coupling to the protein was low at pH values < 4.0 . Immobilisation injections of up to 1200 seconds did not result in any covalent coupling of the protein to the dextran (data not shown), therefore another approach was attempted to immobilise NusA Cterm.

3.2.2.2 NusA Cterm Ligand: Thiol Coupling

The thiol coupling method does not require pre-immobilisation of the ligand before coupling and was therefore thought a suitable method to allow immobilisation of NusA Cterm. Thiol coupling to NusA Cterm was attempted as the protein contains two cysteine amino acids (UniProtKB/Swiss-Prot entry: P0AFF6) that are reduced in the binding buffer due to the presence of a reducing agent (Section 2.3.7). An amine-modified surface was created as detailed in Section 2.5.4. This procedure was carried out to increase the pKa of the surface by converting the dextran carboxyl groups to amine functional groups. After preparation of the amine-modified chip, thiol functional groups were introduced onto the surface using Sulfo-MBS as described in Section 2.5.4. Protein coupling was performed with a 300 seconds injection of 50 $\mu\text{g}\cdot\text{ml}^{-1}$ NusA Cterm prepared in 10 mM sodium acetate pH 4.5, and resulted in an increase of 1180 RU. The surface was then blocked/deactivated using cysteine/NaCl. These chemical and immobilisation steps are shown in Figure 3.15.

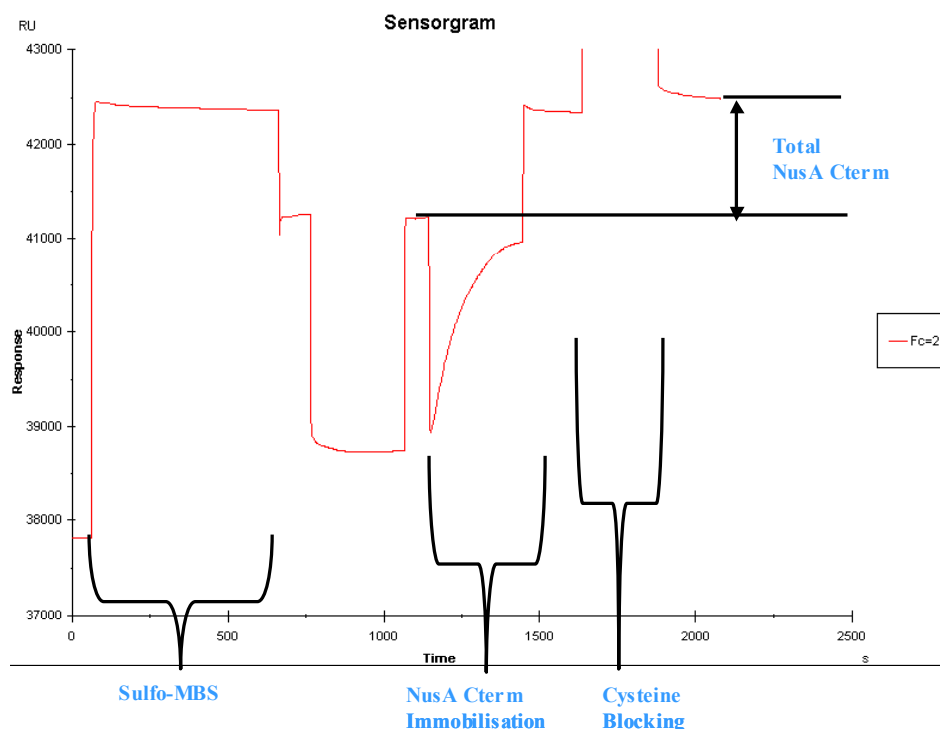


Figure 3.15: Immobilisation of NusA using thiol-mediated coupling to an amide modified CM5 chip.

Each of the phases of the immobilisation cycle are highlighted on the sensogram. ‘Total NusA Cterm’ represents the total amount ~ 1180 RU of ligand covalently attached to the CM5 chip.

Surface tests indicated that injections of 40 μM LamN displayed NSB with the Fc1 ‘blank’. This interaction was removed, however, by supplementing the NusA Cterm/LamN peptide running buffer with 0.005% v/v P20. An injection of 80 μM LamN peptide for 120 seconds was used to estimate the R_{max} of the NusA Cterm surface. Apparent steady state was achieved resulting in an increase of 377 RU. The calculated surface activity using Equation 2.4 was roughly 243%, assuming 1-to-1 binding. The surface activity analysis suggested multiple binding sites on the NusA Cterm immobilised surface, therefore buffer optimisation was attempted to try remove these apparent non-specific interactions.

The LamN peptide has a pI of ~ 12 due to the large number of lysines present in the sequence. It was possible that the LamN peptide was interacting with the negatively charged dextran, even though the surface has been modified with amine groups. The interaction between NusA Cterm and LamN peptide is predominantly charge-charge interactions. It was therefore believed that increasing the ionic strength of the running buffer, while reducing the apparent NSB, could concomitantly alter the kinetic profile of the interaction complex. Addition of biotin ($10 \mu\text{g}\cdot\text{mL}^{-1}$), dextran ($1 \text{ mg}\cdot\text{mL}^{-1}$), 5% DMSO and 10 mM MgCl_2 did not result in a significant decrease in any component of the sensogram (data not shown). As observed for the TrpA/TrpB interaction pair, increasing the amount of detergent in the running buffer to 0.05% v/v did not significantly affect the kinetic response obtained, nor reduce the apparent NSB for the NusA Cterm/LamN peptide interaction. Further attempts to reduce the NSB were not successful. For this reason the source of this NSB was examined further through control binding reactions.

The control for linked-reactions provided evidence for a time dependent component within the binding curve of 10 μM LamN peptide (Figure 3.16). A similar time-dependent dissociation curve was observed using 40 μM LamN peptide (data not shown). A mass transfer control was not attempted due to the steep association curve making comparison difficult, however when analysing low μM affinities using low molecular weight analytes it was not expected to influence the kinetics [111]. The techniques to measure low molecular weight analytes using Biacore has been documented [111, 112].

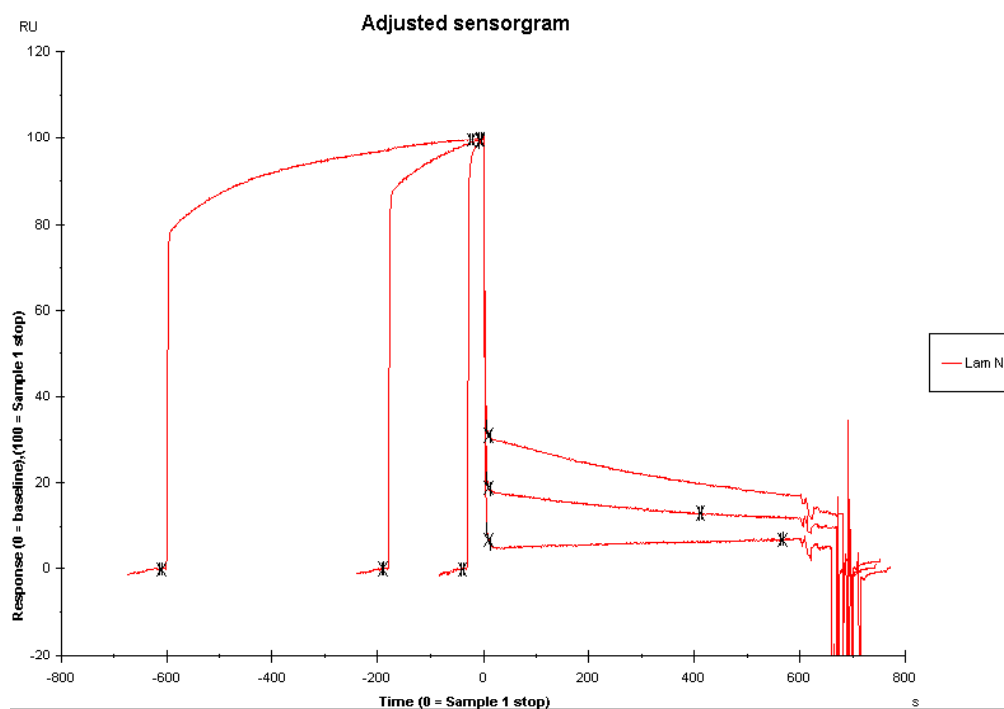


Figure 3.16: Linked reaction control for NusA Cterm/LamN peptide interaction. LamN peptide was injected over the NusA Cterm surface for 30, 180 and 600 seconds and the resulting dissociation profile analysed at a flow rate of $10 \text{ ml} \cdot \text{min}^{-1}$ for changes in the shape of the dissociation curve. The sensorgrams have been adjusted so the association curves were normalised to 100, allowing comparison of sensorgrams that have not reached steady state.

Surface performance was analysed by 10 consecutive cycles of 30 seconds injections of 20 μM LamN peptide followed by regeneration using a 10 seconds injection of 4 M MgCl_2 . A drifting baseline was observed, which could have been due to the limited stability of the amine-modified surface (data not shown). The drifting baseline did not, however, appear to affect subsequent binding cycles as binding remained stable. Hence the surface was deemed suitable for kinetic analysis.

Kinetic analysis generated cycles that could not be adequately fitted to a 1-to-1 model as shown in Figure 3.17. The linked-reaction control suggested a time-dependent conformational change, or additional binding event had occurred, so the data was also fitted to two-state and heterogeneous ligand models as shown in Figure 3.17. To minimise this time-dependent conformational change the injection phase was kept to 30 seconds, therefore steady state conditions were not achieved. It was therefore not possible to use affinity analysis to calculate the K_D of the interaction. The first kinetic run displayed a high χ^2 value of 3.18. This was due to poor fitting of the data

around the injection start and stop points as can be seen in Figure 3.17, resulting in an elevated $R_{\max}$ value Figure 3.17. The mean K_D from the NusA Cterm/LamN peptide interaction using the 30 seconds injections of analyte was $4.51 \mu\text{M}$, which was similar to that measured using ITC (Section 3.1.2.1).

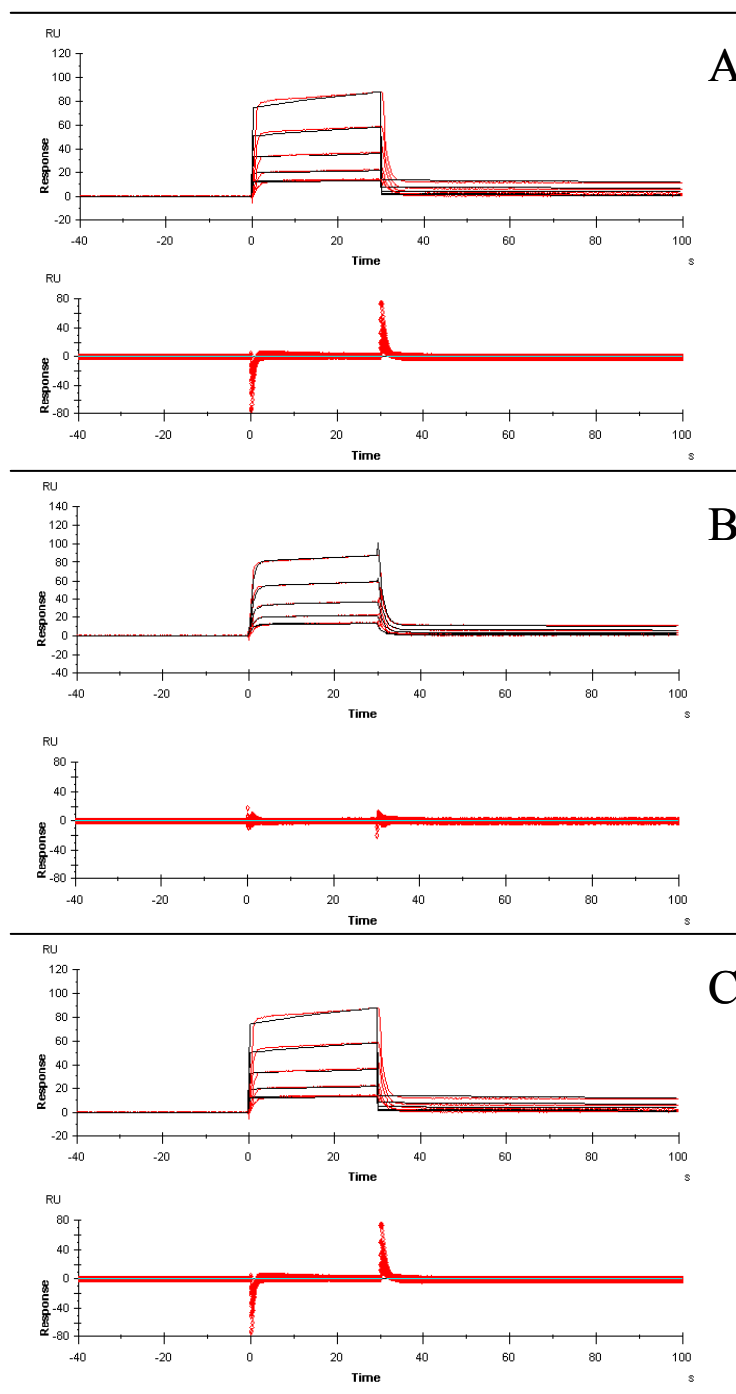


Figure 3.17: Model-fitting to the NusA Cterm/LamN peptide kinetic data.

(A) Sensogram (upper panel) and residual plots (lower panel) for the kinetic data fitted to a 1-to-1 model. (B) Sensogram (upper panel) and residual plots (lower panel) for the kinetic data fitted to a 2-state model. (C) Sensogram (upper panel) and residual plots (lower panel) for the kinetic data fitted to a heterogeneous model. Kinetic reactions were performed using analyte concentrations from $20\text{--}0.625 \mu\text{M}$. Samples were injected for 30 seconds and dissociation was monitored for 600 seconds. Regeneration was performed using a 60 seconds injection of 4 M MgCl_2 and the chip was allowed to stabilise for 240 seconds before the next binding cycle.

Parameter	30 Seconds Injection	T-value	300 Seconds Injection	T-value
ka1 (1·M⁻¹·s⁻¹)	5.51×10^4	150	2.27×10^5	28
kd1 (1·s⁻¹)	0.9240	290	1.755	28
ka2 (1·s⁻¹)	0.003561	350	0.002067	290
kd2 (1·s⁻¹)	0.001308	140	0.001493	700
R_{max} (RU)	281.2	200	197.1	350
K_D (μM)	4.51	-	3.24	-
Chi² (RU²)	0.381	-	1.4	-

Table 3.10: Two-state reaction kinetics to model the NusA Cterm/LamN peptide interaction. The kinetics from 2 independent reactions are shown with different injection times. The two-state model describes a 1-to-1 binding of analyte to immobilised ligand followed by a conformational change that stabilizes the complex ($A+B = AB = AB^*$). It is assumed that the structurally modified complex can only dissociate via the reverse pathway. For this reason the parameters include association and dissociation rate constants for both the binding (ka1 and kd1) and conformational change (ka2 and kd2) ([37]). The overall K_D is determined using Equation 3.4.

Equation 3.4

$$2 - state - K_D = \left(\frac{kd1}{ka1} \right) \times \left(\frac{kd2}{kd2 + ka2} \right)$$

Binding analysis using longer injection times of LamN peptide (300 seconds) was also attempted, in order to analyse how the injection time affected the binding kinetics (Table 3.10). The kinetics obtained for the 300 seconds injection, suggested tighter binding with a K_D of 3.24 μM. This indicated that longer injection times enhanced the extent of the conformational change. To confirm this trend more kinetic assays would have to be performed at longer injection times. For comparison to the solution-based ITC measurements, kinetic data using short injection times was used, in order to minimise the chance of any conformational changes occurring due to experimental artefacts.

Where the association and dissociation rates are too fast to be determined accurately the overall ‘steady state’ binding of the reaction can be analysed to determine the K_D. Equation 3.5 describes the equilibrium dissociation constant K_D for the 1-to-1 interaction from a plot of steady state binding levels (R_{eq}) against analyte concentration (C). RI is a term for the bulk refractive index contribution. After a 300 seconds injection, steady-state conditions (1-to-1) were not achieved, suggesting that the estimate obtained for the steady-state derived K_D of 12.5 μM using Equation

3.5, was probably an over-estimate (Figure 3.18). This suggested that the kinetically derived K_D value of 4.51 μM was closer to the true value.

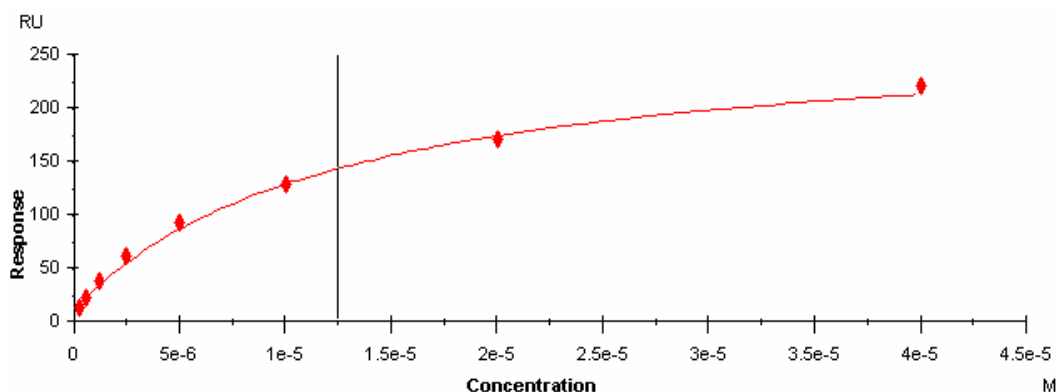


Figure 3.18: Steady-state binding with LamN peptide.
A plot of steady-state binding levels versus the concentration of injected analyte.

Equation 3.5

$$R_{eq} = \frac{(CR_{max})}{(K_D + C)} + RI$$

Where C = analyte concentration

RI = bulk refractive index contribution

R_{eq} = steady state binding level

Without suitable evidence from other structural or spectroscopic techniques, the 2-state model proposed for the NusA Cterm/LamN peptide interaction pair cannot be confirmed. ITC data suggested a 1-to-1 interaction (Section 3.1.2.1). Therefore the data produced via the Biacore model using thiol coupling was not suitable to determine interaction kinetics due to the poor fitting of the 1-to-1 model to the data. This suggested that immobilisation had altered the binding reaction model. The high activity displayed by the surface (> 100% binding) suggested that immobilisation had introduced additional binding sites on the surface, making the surface a poor model. At high analyte concentrations, where the surface is close to saturation, deviations from the 1-to-1 model have previously been reported, as a result of the appearance of second-site interactions on the chip surface [112]. This might explain the poor fitting parameters obtained from the 1-to-1 model, as shown in Figure 3.17, and the reduced χ^2 values of ~ 20 .

Due to the uncertainty over the model selection for the thiol immobilised NusA Cterm sensor chip and the limited methods available to immobilise the protein, the interaction was further analysed using LamN peptide as the ligand.

3.2.2.3 LamN Peptide Ligand: Biotin Affinity Tag Coupling

To confirm the data obtained using immobilised NusA Cterm the binding studies were repeated by preparing a sensor with immobilised LamN peptide. The LamN peptide was synthesized with an N-terminal biotin tag and linker that facilitated its direct

attachment to streptavidin modified chips². ITC data suggested that the addition of the biotin tag did not significantly affect the binding affinity of the NusA Cterm/LamN peptide complex (Section 3.1.2.1). Immobilisation of the peptide through a terminal affinity tag was thought to separate the peptide from the dextran layer allowing free access to the analyte.

Unlike the CM5 chips the streptavidin-modified surface does not require pre-concentration to achieve good rates of protein immobilisation. Due to the tight binding of the streptavidin-biotin interaction low concentrations of ligand were required to produce suitable immobilisation levels for kinetic/affinity analysis.

All 4 flow-cells were used to prepare a surface suitable for kinetic/affinity analysis. Fc1 was used as the blank while varying concentrations of the peptide were injected over the surfaces in Fc2-Fc4. A low, albeit acceptable, level of surface activity was only observed for Fc3, which had significantly more peptide immobilised on the surface than Fc2 and Fc4, as it was very difficult to control the amount of immobilised material at the low response level required. Low level binding to the blank Fc1 was also observed. This NSB was successfully removed by the addition of 0.005% v/v P20 to the running buffer. Increasing the amount of detergent to 0.05% v/v did not significantly affect the kinetic response, or alter the shape of the sensogram, therefore the detergent level was kept at 0.005% v/v.

Using Equation 2.1 an estimation of the activity of Fc3 was ~ 15.5%. As mentioned above, this low activity was not ideal for kinetic analysis. The low surface activity suggested the immobilised peptide was not in an ideal position for binding to NusA Cterm. A peptide with a longer linker between the peptide and biotin tag may have improved the activity of the surface³. Immobilisation of the peptide on the streptavidin chip was difficult to control, and therefore made it hard to get an ideal amount of ligand deposited for kinetic analysis.

As mentioned earlier, a large amount of LamN peptide was immobilised on the surface. The results from the control reaction were therefore important to ensure the surface was suitable for measuring kinetic/steady state binding. Surface performance from Fc3 generated a drifting baseline for over 30 binding cycles with a decreasing $R_{\max}$ and then appeared to stabilise. Stability was observed when 10 binding/regeneration cycles produced a baseline stability of ± 3 RU and a maximum binding of 56 ± 4 RU. Due to the rapid association and dissociation phases and the resulting difficulty in analysing and fitting the data, the mass-transport and linked-reaction controls were not run. Affinity analysis was typically run at $30 \mu\text{l}\cdot\text{ml}^{-1}$, however when analysed at 60 and $90 \mu\text{l}\cdot\text{ml}^{-1}$, no change in the steady state binding level was observed, suggesting mass-transport limitation was not significant for this model.

Estimated dissociation constant:-

The rapid association and dissociation phases as shown in Figure 3.19, did not facilitate the use of kinetic analysis; therefore affinity analysis was used to determine the K_D from the steady-state binding levels using Equation 3.5. Steady-state binding

² SA Certified: BR-1005-31, binding capacity ≥ 1600 RU of a 30-mer biotinylated oligonucleotide

³ The linker contained a 6-carbon spacer between the peptide and the biotin molecule. This information was supplied by Sigma-Genosys.

levels were reached after a 120 seconds injection of analyte for all the concentrations analysed. Model fitting parameters and K_D values from three independent kinetic runs are detailed in Table 3.11. The average K_D from three independent affinity runs was $17.1 \pm 2 \mu\text{M}$. This was significantly higher than that measured using ITC and the value obtained using the 2-state model in Section 3.2.2.2. The change in the observed K_D could have been due to the low activity displayed by the LamN peptide surface and indicated that this was not a suitable model to use for the analysis of the NusA Cterm/LamN peptide interaction. Examination of the T-values for both R_{max} and K_D revealed a low confidence in the derived values. This highlighted the limitation of both the model system and the fit to a steady-state 1-to-1 model.

The problems in developing a suitable Biacore model to analyse the NusA Cterm/LamN peptide interaction suggest that this interaction pair may not be suitable for use as a standard in solid-phase assays. The pI of the NusA Cterm limits the methods that can be used to immobilise the protein, therefore the use of an affinity tag that would allow directed orientation and aid immobilisation would be beneficial for its continued use as an interaction standard.

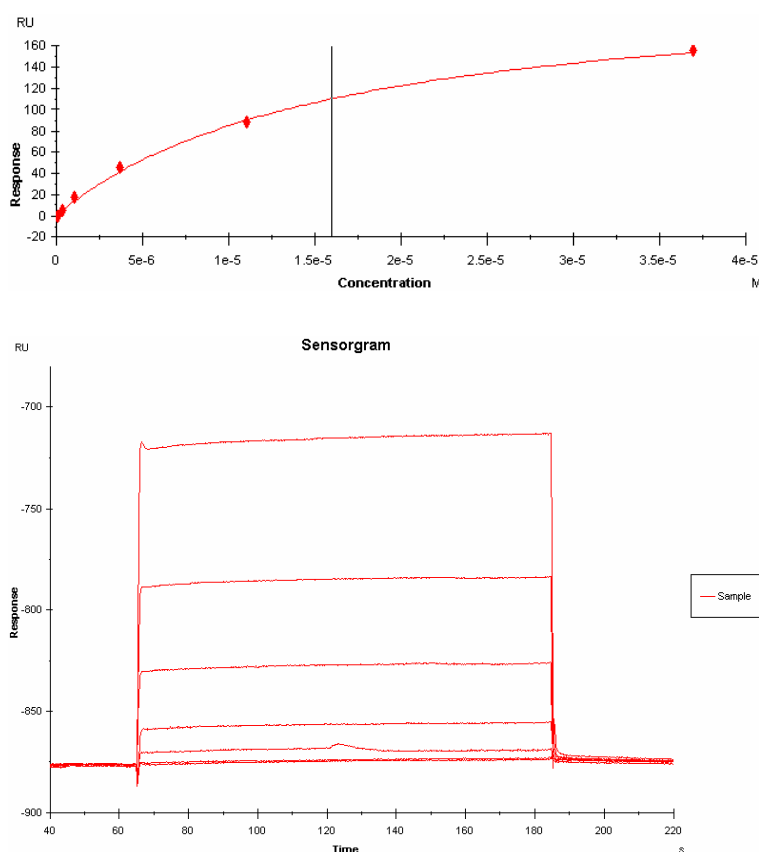


Figure 3.19: Steady-state modelling and sensograms from the NusA Cterm/ LamN peptide interaction.

The top panel shows the steady-state response obtained from the sensogram (lower panel) from a NusA Cterm titration ($37\text{--}0.074 \mu\text{M}$). Data points were taken 5 seconds before the end of the analyte injection when steady-state binding was observed in the association phase. LamN peptide was injected for 120 seconds, dissociation was monitored for 120 seconds and an injection for 60 seconds of 5 M NaCl was used to regenerate the surface. After regeneration the surface was allowed to stabilise for 240 seconds, before starting the next binding cycle.

K_D (μ M)	$T(K_D)$	R_{max} (RU)	$T(R_{max})$	$\chi^2(RU^2)$
16	7.0	220.7	19	12.3
19.4	6.5	220.3	20	9.06
16	8.0	262.1	30	49.3

Table 3.11: Affinity parameters for the NusA Cterm/LamN peptide interaction pair.

3.3 Comparison of Techniques

ITC and SPR are considered orthogonal techniques as they provide information on different aspects of an interacting system. Both measure K_A , but under very different conditions.

Using the two interaction pairs studied in the project, it has been possible to assess the two platforms in terms of ease of use and inter-platform comparability.

ITC is by far the easier of the two techniques to use in terms of experimental design and is also the simplest for data analysis and fitting of a binding model. As the interaction is measured in solution, and in a static “batch” format, none of the many problems associated with surface-based, microfluidics systems are encountered. This means that the data collected using ITC, is often more reliable and hence considered the gold standard. As discussed earlier, there were a number of critical controls required for the SPR measurements in order to distinguish between different binding models and rule out interference from experimental artefacts, such as mass transport. The very nature of the ITC measurements means that there are only heats of dilution controls that are necessary to obtain robust data.

ITC is not high throughput and, relative to SPR, requires a large amount of sample. This is a major disadvantage in the field of drug discovery where many samples to be screened, are only available in small quantities. In contrast once a suitable SPR method has been designed for a given interaction system then SPR is ideally suited for multiple, automated screening measurements.

The K_D of both interaction pairs, measured using the two techniques, is shown in Table 3.12. ITC and SPR gave very similar K_D for the TrpA/TrpB interaction pair. However, as has been discussed in Section 3.2.1.3 the SPR data was shown to best fit to a bivalent model, indicating that avidity interactions were taking place on the chip surface. In contrast the ITC data, where the interaction occurred in solution, showed that only one TrpA subunit was interacting with the TrpB dimer (Section 3.1.1.1). The different binding models suggest that the binding mode for the TrpA/TrpB interaction is somehow changed by the surface attachment of TrpA. This observation highlights a key issue when comparing and interpreting protein-protein interaction data between surface-based and solution-based techniques. Because the structure of a protein is very sensitive to the environment in which it is present, the act of immobilising a

protein to a surface can often lead to changes in structure, which subsequently lead to complete or partial loss in activity.

Measurement Platform	K _D (M)	
	^a TrpA/TrpB	^b NusA Cterm/LamN Peptide
ITC	9.99 ± 1.14 × 10 ⁻⁸	6.35 ± 0.48 × 10 ⁻⁶
SPR	^c 6.68 × 10 ⁻⁸	^d 4.51 × 10 ⁻⁶
		^e 17.1 × 10 ⁻⁶

Table 3.12 Comparison of the K_D values measured using SPR and ITC.

^a All TrpA/TrpB measurements were carried out at 40 °C. ^b All NusA Cterm/LamN peptide measurements were carried out at 25 °C. ^c This is K_{D1} from the bivalent model (Table 3.8). ^d K_D measured using the ligand, NusA Cterm, thiol coupled to a CM5 chip. ^e K_D measured using the ligand, LamN peptide, by means of streptavidin-biotin immobilisation to a SA chip.

The K_D of the NusA Cterm/LamN peptide was measured by SPR using two different types of Biacore chip. The different chips and immobilisation strategies gave different results and are compared to the ITC determined K_D in Table 3.12. The immobilisation strategy using thiol coupling and NusA Cterm as the ligand gave a K_D (value ‘d’ in Table 3.12) very similar to the value measured by ITC. The K_D determined using LamN peptide as the ligand and a streptavidin-biotin immobilisation strategy (value ‘e’ in Table 3.12) was approximately 3 × larger than the K_D determined by ITC. As described in Section 3.2.2.3, the low surface activity may have been responsible for this difference in the K_D.

The data demonstrate that the surface immobilisation process could change the nature of the interaction and, for certain immobilisation strategies, alter the K_D. For this reason it is recommended that more than one immobilisation strategy be used where possible and that the ligand and analyte role be reversed for the interaction measurements. This is often not possible, as was the case here, due to one or both proteins being inactive on one or more surfaces. An alternative approach using antibodies to orientate the ligand molecules during immobilisation is very useful for overcoming a lot of the issues encountered during chemical immobilisation and even his-tag pre-immobilisation steps are useful for preparing more homogeneous, orientated ligand surfaces.

3.4 Proteins Pairs as Potential Interaction Standards

As outlined in the introduction, this project set out to identify a set of protein-protein interaction pairs that could be used as inter-platform comparison standards in the field of protein-protein interaction measurements. While it was not possible to produce genuine standard materials in accordance with the criteria laid out in ISO Guide

34:2000⁴, the interaction pairs described in this report represent an important step towards this goal.

In this report we present robust production and purification protocols for the interaction pairs chosen. Batch-to-batch variation in binding activity has been found to be small. The binding activity of each pair has been shown to be consistent with previous measurements using two orthogonal measurement platforms. The TrpA/TrpB interaction pair was found to be very stable when stored for prolonged periods at -80 °C. This is of course a very important characteristic for a standard material, as it will be required to be stored for long periods of time and still retain its activity. The NusA Cterm/LamN peptide pair was shown to lose activity after extended storage at -80 °C.

No detailed studies into the effect of various additives on the stability of the protein pairs was carried out. Both protein pairs were stored in their respective binding buffers, neither of which contained any cryoprotectant. Most protein products and protein standard materials require the presence of certain excipients in order to stabilise them during storage and any future work would involve testing excipients in order to improve the stability of the NusA Cterm/LamN peptide pair and fully further explore the stability of the TrpA/TrpB pair.

While both interaction pairs were easy to analyse using ITC, both pairs presented problems when studied using SPR. Immobilisation of TrpB using either amine or thiol coupling produced inactive chip surfaces. Only amine coupling produced an active chip surface when TrpA was used as the ligand and this surface-activity was shown to deteriorate after prolonged storage in buffer at 4 °C. However, because the interaction pair was shown to be very stable when stored in solution at -80 °C, and very reproducible surface activity could be generated using the TrpA amine coupling protocols, it is possible that this interaction pair could be used as an interaction standard for SPR.

Generating a suitably active surface for analysing the NusA Cterm/LamN peptide interaction pair by SPR also proved to be difficult. Surface activities were generally low for both the thiol coupled NusA Cterm surface and the streptavidin-biotin immobilised LamN peptide surface. The activity of both of these surfaces was also observed to decrease after prolonged storage at 4 °C.

A set of fully characterised interaction pairs that cover low, medium and high K_D , across the range 10^{-3} to 10^{-11} M, are required. The interaction pairs that have been presented in this report cover the low to mid end of this range as shown in Figure 3.20. A further interaction pair that possesses a K_D in the region of 10^{-9} would prove useful as an inter-platform comparison standard.

⁴ ISO Guide 34:2000, General requirements for the competence of reference material producers (<http://www.iso.org/iso/en/ISOOnline.frontpage>)

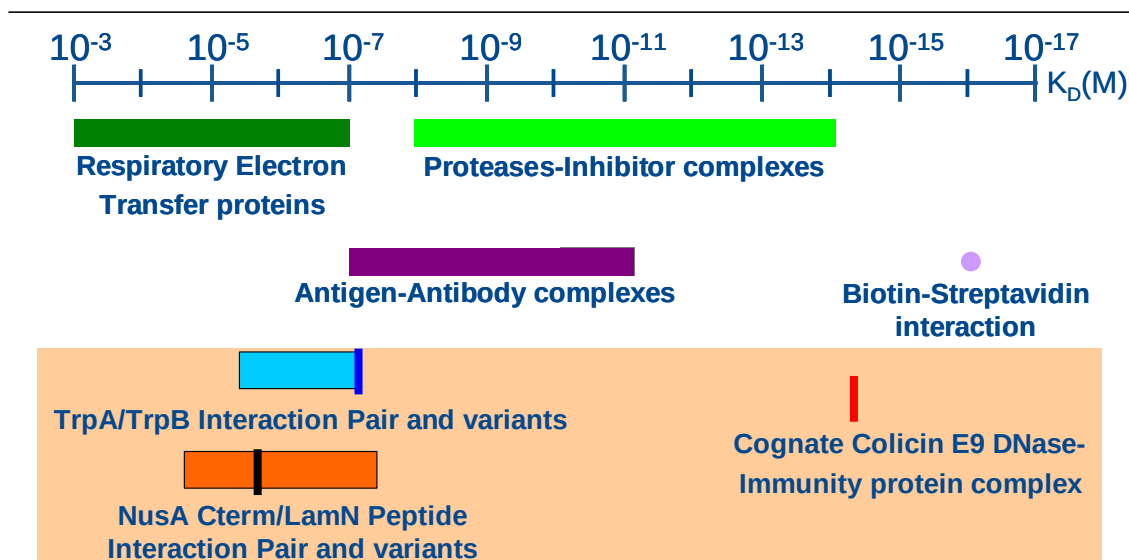


Figure 3.20: Diagram showing the range of K_D the interaction pairs and their potential mutants/variants cover.

Other common protein-protein interaction pairs and families are included as reference points.

There is interest elsewhere in the development of protein-protein interaction standards for the purposes of instrument validation and inter-platform comparison. The Molecular Interactions Research Group (MIRG), part of the Association of Biomolecular Resource Facilities (ABRF), is a virtual research group made up of a number of research institutions and biopharmaceutical companies. The members of MIRG have been working towards a protein-protein interaction standard based on the barnase/barstar protein complex. This interaction pair has a number of characteristics that make it suitable as a potential interaction standard and two mutants of barnase and one of barstar have already been produced which have altered the binding strength of the interaction [113]. Complexes between the wild-type and variants of the two proteins are said to cover a range of K_D from 10^{-14} to 10^{-6} and therefore have a suitably large dynamic range to potentially act as protein-protein interaction standards. The approach taken by MIRG, where a single well-characterised protein pair with very tight binding is selected, is promising. Mutants can then be produced that modify the binding affinity, thus providing a number of interaction standards from only a single protein pair. Early on in the selection of our interaction pairs we also applied the same approach, however, of all the interaction pairs that were screened, only the Bacterial Colicin E9/Immunity Protein complex had suitably tight binding ($K_D \sim 10^{-14}$ M) (Figure 3.20). Unfortunately, expression and purification of the cognate pair proved extremely difficult and prevented it from being taken any further.

4 Conclusions

This report has described the work carried out towards the development of two protein-protein interaction pairs as interaction standards, and their evaluation using ITC and SPR. The binding affinities of both interaction pairs were successfully measured using both ITC and SPR and the results obtained were found to be consistent between the two techniques and with those reported by other researchers.

The suitability of the two interaction pairs as potential protein-protein interaction standards, however, remains to be fully assessed. Of the two interaction pairs, the TrpA/TrpB pair is by far the more suitable system due to its excellent stability during storage and the reproducibility of the binding measurements obtained.

The work carried out in this study has confirmed that the selection, production and characterisation of interaction inter-platform comparison standards is far from simple. This is due to the diverse nature of protein-protein interactions, the technical difficulties involved in producing stable binding pairs and the broad measurement requirements of the various platforms.

If the stability of the NusA Cterm/LamN peptide pair can be improved then more work is required to fully characterise the thermodynamic binding parameters of both interaction pairs using ITC and SPR. Comparison of these parameters, measured using the two techniques, would allow further insight into the effects of surface immobilisation on the accurate measurement of protein-protein interactions.

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A Appendix

A.1 TrpA Purification Chromatograms

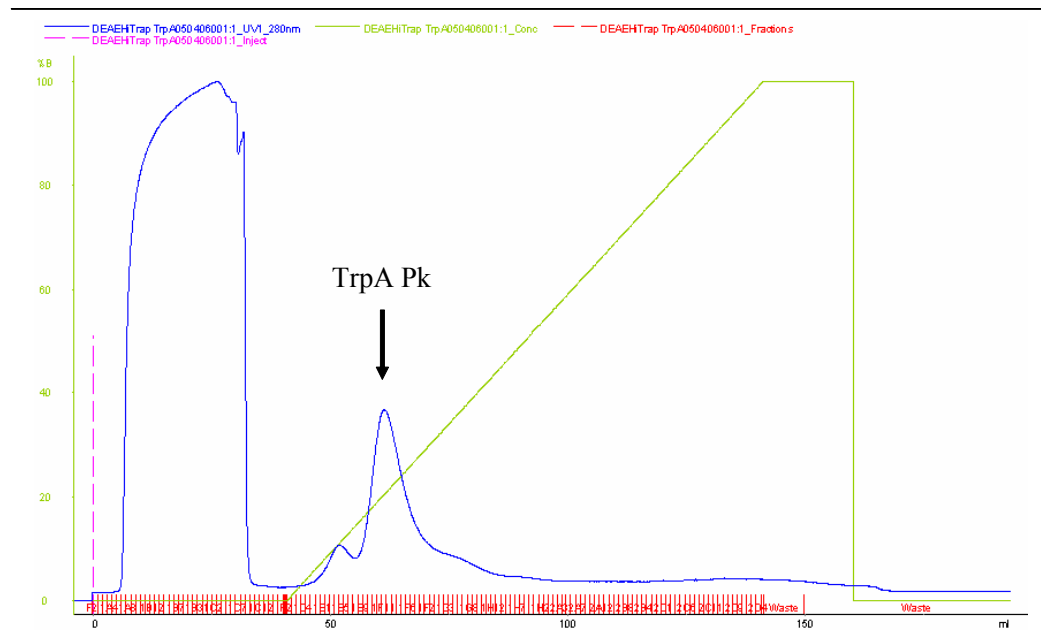


Figure A.1: HiTrap DEAE column chromatogram for TrpA.

The peak corresponding to TrpA is indicated. A 5 ml HiTrap DEAE FF column (GE Healthcare) equilibrated in 5 mM potassium phosphate pH 7.0 at 4 °C was used. The column was developed at 2.5 ml·min⁻¹ and the protein of interest was eluted at ~ 20 mM potassium phosphate using a 20 CV linear gradient of 5–100 mM potassium phosphate pH 7.0.

A.2 TrpB Purification Chromatograms

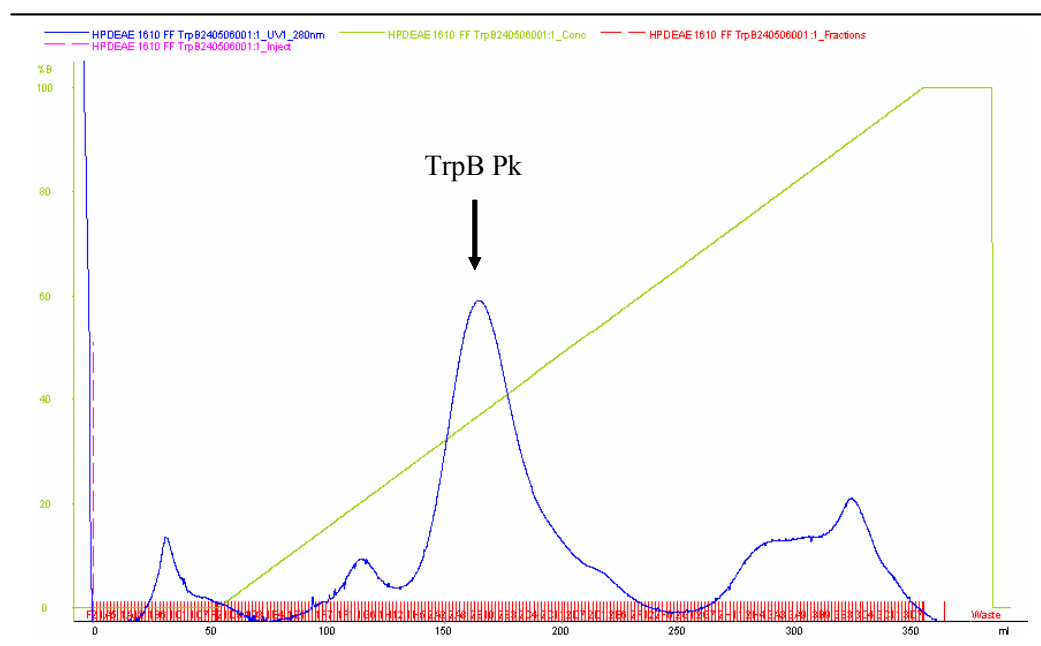


Figure A.2: HiTrap DEAE column chromatogram for TrpB.

The peak corresponding to the elution of TrpB is indicated. A 5 ml HiTrap DEAE FF column (GE Healthcare) equilibrated in 100 mM potassium phosphate pH 7.0, 1 mM DTT, 5 mM EDTA, 0.02 mM PLP at 4 °C was used. The column was developed at a flow rate of 2.5 ml·min⁻¹ and TrpB was eluted at ~185 mM NaCl in a 20 CV linear gradient of starting buffer containing 0-500 mM NaCl.

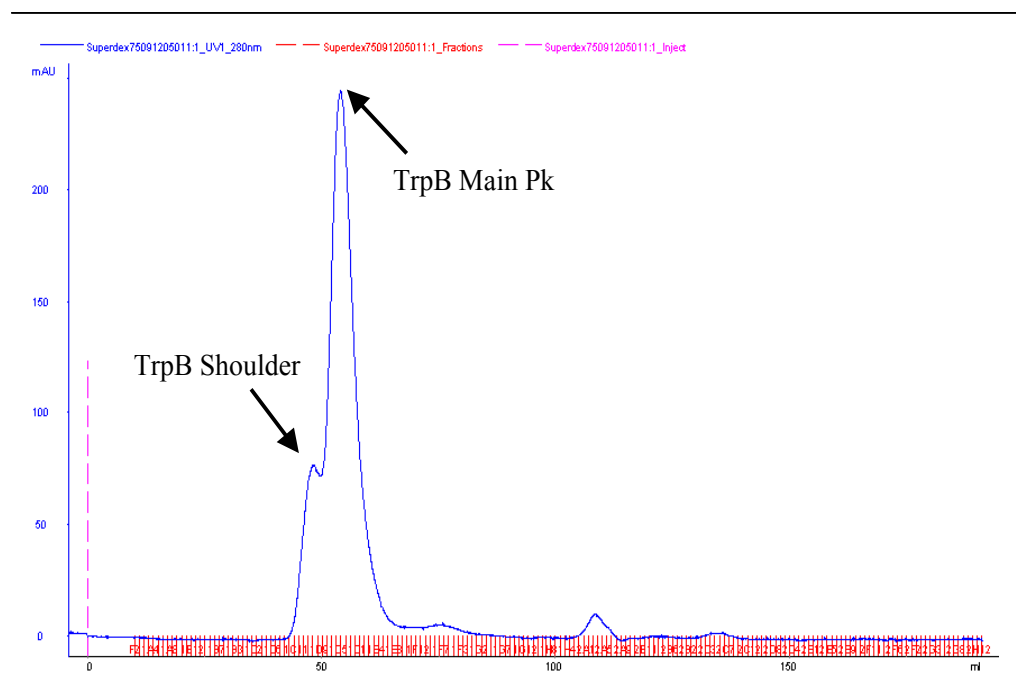


Figure A.3: Superdex 75 16/60 column chromatogram for TrpB.

The main peak and shoulder corresponding to TrpB are indicated. A Superdex 75 16/60 column (GE Healthcare) equilibrated in 50 mM potassium phosphate pH 7.0, 300 mM KCl, 0.1 mM DTT, 5 mM EDTA, 0.02 mM PLP was used. This column was developed at 0.5 ml·min⁻¹ and the eluted fractions monitored at 280 nm.

A.3 NusA Cterm Purification Chromatograms

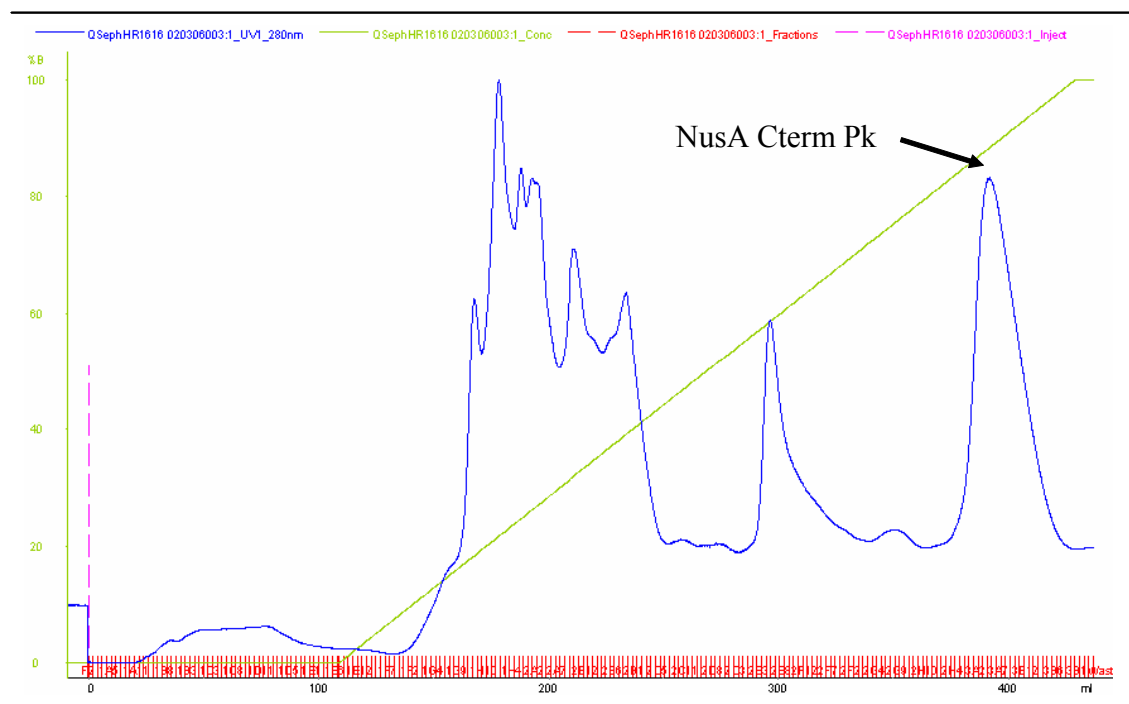


Figure A.4: HiTrap Q FF column chromatogram for NusA Cterm.

The peak corresponding to NusA Cterm is indicated. A 5 ml HiTrap Q FF column (GE Healthcare) equilibrated in 5 mM potassium phosphate pH 7.0 and developed at a flow rate of 2 ml·min⁻¹. The protein of interest was eluted at ~880 mM potassium phosphate using a 10 CV linear gradient of 0.005-1 M potassium phosphate pH 7.0

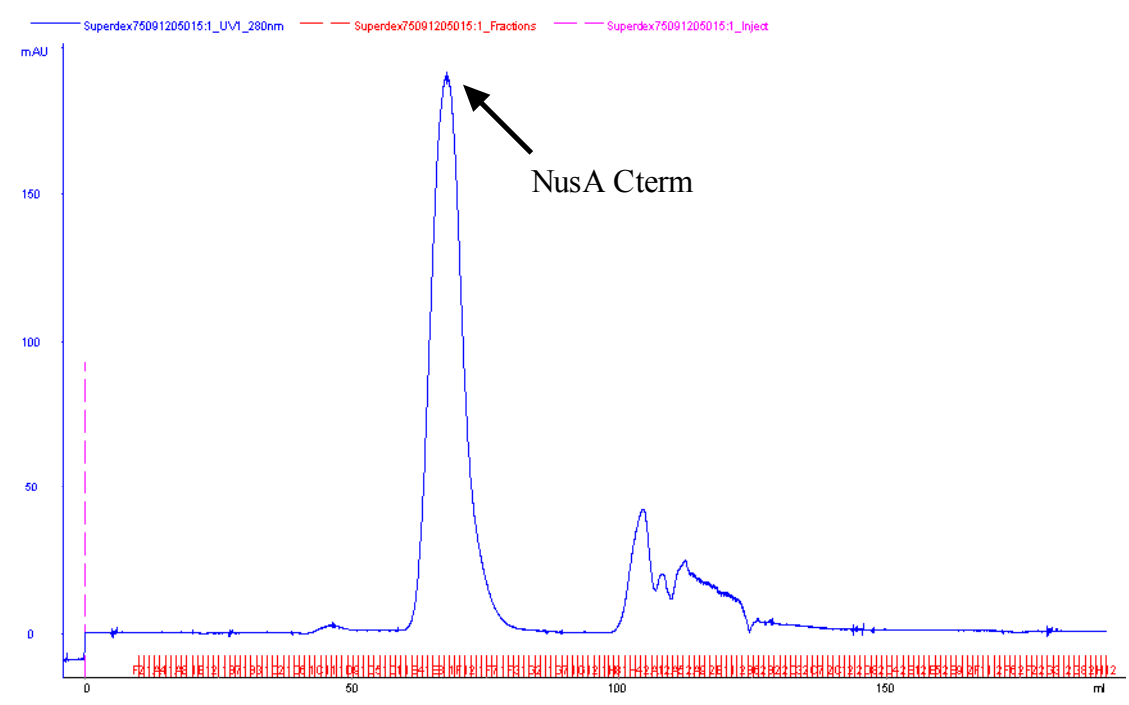


Figure A.5: Superdex 75 16/60 column chromatogram for NusA Cterm.

The main peak corresponding to NusA Cterm is indicated. A Superdex 75 16/60 column (GE Healthcare) equilibrated in 50 mM Tris pH 7.0, 150 mM NaCl, 2 mM DTT and developed at 1 ml·min⁻¹ was used. The eluted fractions were monitored at 280 nm.