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Molecular recognition of peptides.

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MOLECULAR RECOGNITION OF PEPTIDES

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Dedicated in memoriam

My parents, Joyce and Bryn Grail

Cledwyn R. Jones

Mick Burrill

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ABBREVIATIONS

Standard three letter amino acid codes are used.

3DPR	three-dimensional pseudo-Ramachandran
3DR	three-dimensional Ramachandran
3DVT	three-dimensional virtual torsion
ABC	ATP-binding cassette
Ac_2KDADA	di-acyl-L-Lys-D-Ala-D-Ala
Ac_2KDADLac	di-acyl-L-Lys-D-Ala-D-Lactate
Ac_2KDADS	di-acyl-L-Lys-D-Ala-D-Ser
ATP	adenosine 5′-triphosphate
Dpp	dipeptide permease
DppA	dipeptide binding protein
FPLC	fast protein liquid chromatography
IEF	iso-electric focussing
ITC	isothermal titration calorimetry
MRT(s)	molecular recognition template(s)
MSS	molecular spreadsheet
NAG	*N*-acetylglucosamine
NAM	*N*-acetylmuramic acid
Opp	oligopeptide permease
OppA	oligopeptide binding protein
PBP	penicillin binding protein

QSAR	quantitative structure-activity relationship
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SPL	SYBYL programming language
Tpp	tripeptide permease
VT	virtual torsion angle

SUMMARY

Computer-based molecular modelling was combined with biochemical and biophysical methods to determine the structural features of peptides that are important for their recognition by peptide transporters. Information gained from this study advances the understanding of ligand recognition in general as well as by peptide transporters in particular.

A stochastic search protocol, using implicit solvation, was used to generate conformer sets for di-, tri- and oligopeptides. Dedicated programs were written to analyse these conformer populations and the results displayed using novel 3D plots. Contrary to current perception our results show that small peptides adopt a limited number of backbone conformations in solution. Combining information on these predominant backbone forms with results from peptide transport assays on individual peptides defined the molecular recognition templates (MRTs) for Dpp and Tpp. The relationship between the proportion of MRT conformers and the relative affinity of a peptide for a transporter showed Dpp and Tpp to be kinetically-driven. The ability of tripeptides to act as substrates for these transporters was explained by the presence of 'folded' forms that matched the dipeptide MRT. Extended tripeptides and oligopeptides comprised a subset of backbone forms that are substrates for Opp, highlighting the complementarity of the three archetypal bacterial peptide transporters. Information upon the MRTs of peptide transporters will greatly assist the rational design of smugglin type drugs designed to exploit these transport systems for their delivery.

Methodology devised here was also applied to the study of the recognition of cell-wall peptides and β-lactam antibiotics. The information gained on the conformations adopted by each of these compounds suggested features important for their recognition by transpeptidases and gave further insight into the inhibitory action of β-lactam antibiotics. Cell-wall peptide analogues, found in most vancomycin resistant strains, were also modelled and shown to have a different distribution of conformational types that may be an important factor in resistance to glycopeptide antibiotics. Knowledge of the MRTs of transpeptidases and vancomycin will aid the development of new antibacterial compounds and further our understanding of antibiotic resistance mechanisms.

I. RECOGNITION IN BIOCHEMICAL REACTIONS

A fundamental aspect of biochemical processes is high affinity and specificity in molecular recognition. At its simplest, molecular recognition can be defined as the specific interaction of two, or more molecules, to form a productive (long-lived) complex. Structural and electronic features within each molecule contribute to both the specificity and affinity, providing consolidating interactions that ensure the correct species combine and that a stable complex is formed. Collisions between molecules that do not possess the correct features for recognition cannot be consolidated in this manner, and so will be transient. This broad definition of molecular recognition is unmanageable and so a more focussed viewpoint is taken by only considering the specific interaction between small organic molecules (substrates, ligands) and proteins (enzymes, receptors or transport proteins).

Recognition of ligands by proteins

Affinity is a measure of the strength of interaction between two molecules and is quantified by the equilibrium constant for the association reaction (K_F), which for an interaction between a single binding-site protein and a ligand can be represented by equation ①.

①

$$E + S \underset{}{\overset{K_F}{\rightleftharpoons}} ES$$

E = Protein S= Ligand
ES = Protein/Ligand complex.

The equilibrium constant for the formation of the ES complex is $K_F = [ES] / [E] [S]$, and is identical to the term K_B used for binding reactions. The dissociation constant

of the ES complex is K_S, which has an inverse relationship to K_F and is equivalent to K_D for binding reactions and (equation ②) (Fersht, 1977).

$$K_B = \frac{1}{K_D} \quad K_F = \frac{1}{K_S} \qquad ②$$

For molecules that interact with a high affinity, K_B or K_F is large, i.e. K_D or K_S is small. The specificity of the interaction is an indication of how discriminating a protein is between a set of different ligands and will usually have to be qualified by some indication of the ligand types considered.

Study of protein/ligand interactions

The study of molecular recognition has taken two main routes; structural determination of the protein (E) or protein/ligand (ES) complex and approaches centred upon the structural or physico-chemical properties of the ligand (S), the former being the more prevalent. Protein structure elucidation is dominated by X-ray crystallography, the quantity and quality of crystal structures having increased dramatically over the last few years. Crystallographic data can provide remarkable 3-dimensional displays using modern molecular graphics rendering, allowing the viewer to almost move within the structure. Where crystal structures of target proteins have been obtained with bound ligands, attempts to characterise the binding site can be made. The correct identification of a ligand-binding site within a protein crystal may be extremely difficult, and is compounded by any conformational changes a protein may undergo upon ligand binding. DppA is a good example, where in the "open"; unliganded form, the Asp^{408} and Arg^{355} side chains involved in binding the charged termini of dipeptide substrates are much further apart compared

with their position in the "closed"; liganded form (Dunten and Mowbray, 1995; Nickitenko *et al.*, 1995). However X-ray crystallographic studies are limited by a requirement for purified protein, and the difficulties associated with co-crystallisation of a protein with ligand. For many potential targets, such as integral membrane proteins, crystallography still remains nearly impossible at present.

Biochemical studies of protein-ligand complexes are an alternative approach to understanding molecular recognition. Techniques used to quantify specific complex formation range from relatively simple competition binding assays to sophisticated isothermal titration calorimetry (ITC) measurements. Competition assays give a relative measure of binding (K_D), as well as ranking different substrates according to affinity (Smith *et al.*, 1999). ITC provides good measurements of affinity (K_B), as well as quantifying thermodynamic elements of the complex formation.

In light of the difficulties of protein-centred methodologies, investigating molecular recognition by using a ligand-based approach is an attractive idea that is not new to the field. This approach depends upon identifying the structural and conformational features that are required for recognition and are shared by all good substrates. Quantitative structure-activity (QSAR) studies use substrates (active analogue approach), as well as inactive compounds, to identify the common structural features that correlate with a particular activity. Such studies result in the identification of bioactive conformations or provide pharmacophore descriptions, both of which have become central to understanding structure-activity relationships.

Descriptors of peptide molecular surfaces

Bioactive conformations are a theoretical notion, being the substrate conformation that is actually bound and brings about a physiological response. This concept of a

single active conformation focuses upon individual minimum-energy or low-energy conformations and may tend to overlook the conformer population as a whole. Studies trying to relate recognition to particular consolidating intermolecular interactions have, as a necessity, tended to regard the substrate as a single bioactive conformation (Davies *et al.*, 1999; Sleigh *et al.*, 1999).

For bimolecular interactions to be productive, i.e., elicit a biological response, they have to take part in the essential first step of molecular recognition. Simplistically, this recognition event can be viewed as the coming together of complementary 3D surfaces on both the protein and ligand to form a complex that is consolidated by intermolecular interactions such as H-bonding, salt-bridges and hydrophobic stabilisation. The portion of a ligand's 3D surface that is complementary to the binding site is referred to as a pharmacophore. For peptide ligands, the pharmacophoric surface is generated within the conformational space occupied by the amino acid side chains, and can be made from a contiguous or non-contiguous primary sequence. The peptide backbone itself functions as a scaffold for the key side chain groups, their steric and electrostatic properties providing the correct surface shape for efficient molecular recognition. The 3D surface presented to the protein by peptide ligands (2-5 amino acids) is predisposed by their backbone conformation. The influence side chain chemistries and/or conformation have upon the initial recognition event will be influenced by the function-determined specificity of the protein. Highly specific proteins (e.g. peptide hormone receptors) will evolve to exploit the diversity of side chain chi (χ) space, while those with broad substrate specificity (e.g. peptide transporters) are more likely to recognise shared structural features that are predominantly determined by backbone (ψ, ω, ϕ) conformation.

Comparison of activity data, such as that obtained from competition binding assays, for a range of selected substrate analogues will usually allow simple pharmacophoric patterns to be identified e.g. peptides require a positively-charged amino terminus for recognition by OppA (Payne and Gilvarg, 1971). It is clear that pharmacophores are only an outline definition of any recognisable conformation and may fail to shed any light upon why two peptides containing the same pharmacophore have different activities.

Recently, the term "molecular recognition template" (MRT) has been introduced to provide a more detailed description of the optimal structural features required for recognition than is achieved by either the bioactive conformation or pharmacophore (Payne *et al.*, 2000b). Molecular recognition templates are multi-feature descriptions of structural, conformational, electrostatic and stereochemical parameters that are required for optimal recognition of a ligand by its cognate macromolecule. Effective recognition of good substrates is achieved with solution conformers having optimal MRT features. Compounds that lack the relevant forms, or possess MRT features that are outside the optimal range will be poorly recognised and may not be considered as substrates. The lack of critical MRT features, such as correct stereochemistry or charge, can result in a marked loss, if not abolition, of activity. One of the main elements of the MRT that has been explored by Payne's group in Bangor is the impact that the backbone conformer profiles of peptide substrates have upon substrate specificity and evolution of peptide transport systems (Payne *et al.*, 2000a, 2000b and 2002).

[The MRT concept and the role of peptide MRTs in driving peptide transporter evolution are discussed in paper (i).]

II. Substrate specificities of peptide transporters

Peptide transport systems, of which the bacterial systems are the best characterised, have been identified in all species studied to date (Payne and Smith, 1994; Payne and Marshall, 2001). *E. coli* and *S. typhimurium*[*] each possess three main systems; the dipeptide (Dpp), tripeptide (Tpp) and oligopeptide (Opp) transporters. Dpp and Opp are multi-protein ABC transporters, their substrate specificity being dictated by the periplasmic binding proteins DppA, OppA, respectively. Tpp is a single protein system energised by a proton motive force and so lacks a periplasmic binding protein.

Structural features of peptides

Pioneering work on peptide transporters started to define the structural features required for recognition and transport by prokaryotic and eukaryotic peptide transporters (Matthews, 1991; Payne and Smith, 1994). These early studies determined many pharmacophoric recognition elements using the experimental systems available at the time. Later, purification of DppA and OppA allowed relative binding affinities (K_D) to be measured for a range of substrates (Guyer *et al.*, 1986; Smith *et al.*, 1999; Tyreman, 1990; Tyreman *et al.*, 1992). Peptides have characteristic structural features that form the basis for peptide substrate recognition.

Ionisable N- and C- termini

Peptides at physiological pH values (~7) will be zwitterionic carrying a positively charged N-terminal α-amino group and a negative C-terminal α-carboxylate. Although the proportion of protonated N-terminal α-amino groups existing at

[*] Now called *S. enterica* serovar Typhimurium

particular pH values will vary slightly depending upon its pKa, measurement of ligand binding to DppA showed little change in binding over the pH range 3.0 - 9.5. The paramount importance of a protonated α-amino group for transport by bacterial peptide transporters is well documented (Payne and Gilvarg, 1971; Smith *et al.*, 1999).

Charged C-terminal α-carboxylate groups are also important for recognition of di- and tripeptides by Dpp and Tpp. In contrast, Opp can still recognise peptides with modified C-terminal carboxylate groups, e.g., esters, albeit less well, a feature presumably related to its broader specificity.

As well as carrying charge, the N- and C-termini, along with the resonant peptide bond, have considerable hydrogen bonding potential. This, particularly in the case of the peptide bond, is made use of in the binding mechanisms of both DppA and OppA, where hydrogen bonding of the peptide backbone to the protein occurs (Dunten and Mowbray, 1995; Tame *et al.*, 1994).

Stereochemistry

Peptides derived from protein hydrolysis are composed of L-amino acid residues. Only in certain circumstances e.g. bacterial cell wall recycling or bacterial infection, will peptides containing D-amino acids form part of the substrate pool. The stereochemistry of the Cα in amino acid monomers imposes a particular orientation of the four attached groups, which is important in terms of the side chain position.

Backbone torsion angles (ψ, ω, ϕ,)

The peptide backbone can be considered as a set of connected torsion angles. Omega (ω) is the torsion angle about the peptide bond (Cα-C′-N-Cα), the chemical characteristics of which result in a resonance-stabilised structure (Fig.1). This has

two main forms, *cis* and *trans*, the values for omega being 0 ° ± 5 ° and 180 ° ± 5 °, respectively. Either side of the omega bond are the psi (ψ) and phi (ϕ) torsion angles, which are defined by N-Cα-C′-N and C′-N-Cα-C′, respectively (Fig. 1). Unlike the resonance-constrained omega torsion, both these torsion angles are able to rotate freely in peptides except where the constrained amino acid residues proline and pyroglutamic acid occur. Consecutive numbering of peptide backbone torsion angles allows identification of each individual rotatable bond (Marshall *et al.*, 2001). For dipeptides the ψ angle is Tor 2 and the ϕ angle is Tor 4 (Fig. 1).

Side chain torsion angles (χ)

With the exception of Gly and Ala, the amino acids commonly found in proteins have side chains containing one or more χ torsion angles, which are mainly found in three forms, *gauche* (+) 60 ° (g^+), *gauche* (-) −60 ° (g^-) and *trans* ±180 ° (*t*) (Fig. 1).

N to C (N-C) *distance*

The distance (N-C) between the N-terminal α-amino nitrogen and the C-terminal α-carboxylate carbon expresses a measure of charge separation in peptides. This distance is determined by the backbone torsion angle combinations of peptides and has been used to try to define structural recognition features.

III. Assays for peptide transport and binding

A range of assays was developed, based upon biochemical and biophysical techniques, to quantify the ability of peptides to act as peptide transport substrates.

To achieve accurate measurements of peptide uptake or competition, ideally cells having single, defined uptake systems are required. These studies initially used

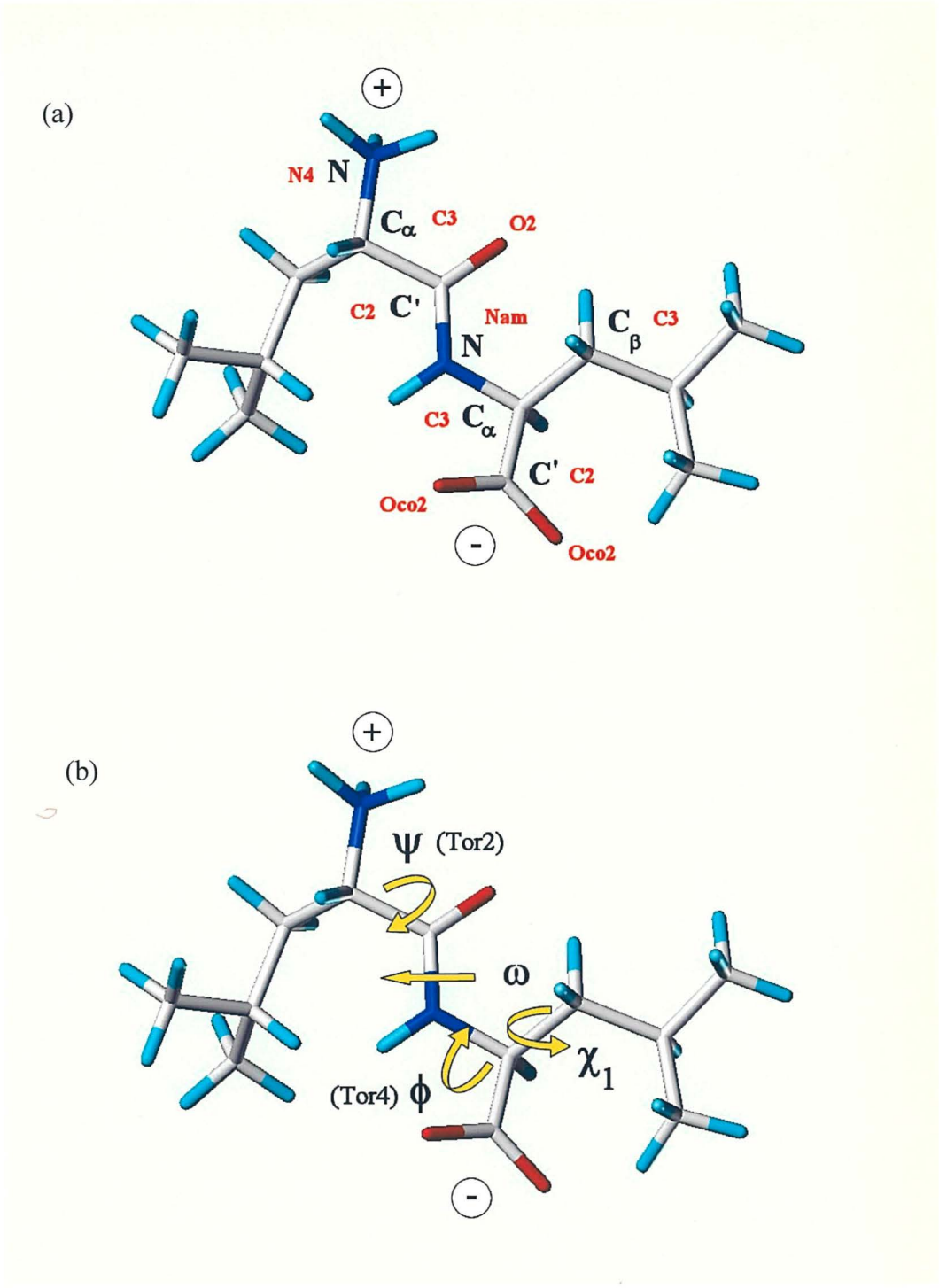

Figure 1. Backbone and side chain atom and torsion angle definitions of a dipeptide (LeuLeu)

The conventional peptide backbone atom nomenclature (black) and example SYBYL atom types (red) are given in (a). Torsion angles within the dipeptide backbone and side chain are annotated with conventional Greek symbols and indicated by arrows (b).

E. coli W strain M26-26, which is a lysine auxotroph, and later *E. coli* K-12 strain Morse 2034 (CGSC5071), which is a Leu and Trp auxotroph and, as with all K-12 strains, is valine sensitive (Payne and Smith, 1994). A mutant (PA0183) deleted for *opp* was selected from Morse 2034 and used as a parent for further selection of mutants using combinations of permease-specific inhibitory peptides and antimicrobial smugglins (Table 1.) (Smith *et al.*, 1999).

Assay for uptake of peptides

Payne and Nisbet developed a method to assay the uptake of peptides based upon the reactivity of the unprotonated α-amino group with fluorescamine (Nisbet and Payne, 1979; Payne and Nisbet, 1980). Measuring the disappearance of peptide from incubation medium allowed monitoring of its uptake by a suspension of washed bacterial cells (Payne *et al.*, 2000a; Smith *et al.*, 1999). Samples of incubation medium were taken at timed intervals and the cells removed immediately by filtration through glass fibre filters. The cell-free incubation medium was then assayed for peptide by reaction with fluorescamine in borate buffer pH 6.2 (Nisbet and Payne, 1979; Payne and Nisbet, 1980). Fluorescamine reacts with picomole quantities of primary amines to form fluorescent derivatives. Perrett *et al.* described the pH dependence of this reaction and showed that although there was a marked reduction in sensitivity compared with reaction at pH ~9 (~70%), the maximal difference in reactivity between small peptides and amino acids occurred at pH 6 (Perrett *et al.*, 1975). The greater reactivity of small peptides at this pH can be attributed to a lower pKa for their α-amino group. Therefore performing the reaction at pH 6.2, allows the assay of peptides with negligible interference from amino acids. Linear time courses of peptide disappearance provided initial rates of uptake, which

Strain	Genotype	Source	Pedigree	Supplements	References
E. coli W					
M26-26	*lys*	Prof. C. Gilvarg Princeton Uni. U.S.A.	Derived from ATCC 9637	Lys	Payne and Gilvarg (1968)
E. coli K-12					
Morse 2034	*trpE*9851, *leu*277, IN *(rrnD-rrnE)*1, λ^-	*E. coli* Genetic Stock Centre, Yale Uni., U.S.A.		Leu, Trp	Morse and Guertin (1972)
PA0183	*Δ(tdk-tonB)*	This lab	Derived from M2034	Leu, Trp, Fe^{3+}	Smith *et al.* (1999)
PA0333	*Δ(tdk-tonB), dpp*	This lab	Derived from PA0183	Leu, Trp, Fe^{3+}	
PA0410	*Δ(tdk-tonB), tpp*	This lab	Derived from PA0183	Leu, Trp, Fe^{3+}	
PA0643	*Δ(tdk-tonB), dpp, tpp*	This lab	Derived from PA0333	Leu, Trp, Fe^{3+}	
PA0610	*Δ(tdk-tonB), dpp, tpp*	This lab	Derived from PA0410	Leu, Trp, Fe^{3+}	
JM101	*supE, thi, Δ(lac-proAB)* [*F'traD36 proA*$^+$ *proB*$^+$ *lac1*^{q}*ZΔM15*]			Thiamine	Abouhamad and Manson (1994) Olson *et al.* (1991)

Table 1. Bacterial strains used in these studies.

when expressed per unit amount of cell allows comparison of the relative rates of uptake (Payne *et al.*, 2000a; Smith *et al.*, 1999).

Competition plate assays

Valine-containing dipeptides produce inhibition zones when applied to paper disks on agar plates of growing *E. coli* K-12 strain Morse 2034. Using Val-containing dipeptides (e.g. ValGly, ValPro) as inhibitors on a central paper disk, allowed the relative affinities of test peptides to be judged from their abilities to compete. In a modification to the standard agar plate diffusion assay an equal amount of competing peptide was placed on paper disks at different distances from the central inhibitor disk. This allowed the gradient of competing peptide to be accurately related to the disk separation so improving the assay's accuracy (Payne *et al.*, 2000a). This simple assay made it possible to screen a large number of peptides as potential peptide transporter substrates and rank them according to their affinity. Results from such assays correlated well with transport and binding studies, allowing a dataset of relative activities to be correlated with modelling data. For Tpp, other than assaying transport directly, it was the only assay available as the transporter is yet to be purified.

Purification of periplasmic peptide-binding proteins

The periplasmic peptide-binding proteins, DppA and OppA, are easily purified in milligram amounts from *E. coli* strains, Morse 2034 or JM101 by cold osmotic shock treatment followed by FPLC cation-exchange chromatography (MonoS). Further purification by reverse phase (C18) HPLC in an aqueous acetonitrile / trifluoroacetic

acid gradient buffer system produces pure, unliganded, protein which can be used for assays where ligand-free protein is critical (Payne *et al.*, 2000a; Smith *et al.*, 1999).

Filter binding assays

Filter binding assays with DppA showed that the substrate specificity of Dpp was determined by the binding affinities of the periplasmic binding protein (Smith *et al.*, 1999). Direct measurement of peptide binding was obtained by incubating DppA with ^{14}C-radiolabelled peptide (AlaPhe, GlySar or GlyLeu) and quantifying the amount of labelled peptide retained on a filter after filtration and washing of the ammonium sulphate precipitated DppA-peptide complex (Payne *et al.*, 2001; Smith *et al.*, 1999). However, the application of this method is limited by the availability of high specific-activity radiolabelled peptides. To determine binding for a range of peptides, competition filter binding assays provide a convenient alternative. Purified DppA was incubated with [^{125}I]GlyTyr, in the presence of a competing unlabelled peptide. The displacement of radiolabelled ligand was quantified by gamma counting the ammonium sulphate precipitated protein-ligand complex. Comparing different competing peptides at constant protein to ligand ratios allows one to rank substrate affinities in a relative manner. Analogous competition filter binding assays can be carried out with purified OppA using an iodinated tripeptide e.g. [^{125}I]TyrGlyGly as substrate (Payne *et al.*, 2000b; Smith *et al.*, 1999).

Isothermal titration calorimetry

The specific binding of two molecules results in a free energy change (ΔG_B °) that has enthalpic (ΔH_B °) and entropic (ΔS_B °) contributions. Isothermal titration calorimetry (ITC) is a technique that quantifies the thermodynamic components of

this interaction by accurately measuring the total heat output/input required to maintain the temperature of a binding reaction incubation constant (Ladbury and Chowdry, 1996). ITC directly quantifies thermodynamic contributions, characteristic to a biomolecular interaction, in a non-destructive manner. Coupling this information with detailed structural information offers much promise in the calculation of binding parameters that are of use to the pharmaceutical industry (Ladbury and Chowdry, 1996).

[The results of using competition plate assays, filter binding assays and ITC to determine the biological activity of a range of dipeptides can be found in papers (i), (iii), and (iv).]

IV. CONFORMATIONAL ANALYSIS OF PEPTIDES

The aims of this study were well defined at its conception - to use molecular modelling to produce a reasonable representation of the conformational forms adopted by simple, charged peptides in solution and to determine any shared conformational features. The results from these modelling studies would be compared with the biological data for each peptide transporter to determine the features important for recognition.

Conformational searching

Earlier studies tended to model peptides as portions of protein structure using acylated and amidated peptides as model compounds (Cornell *et al.*, 1997; Marshall *et al.*, 1993; Nikiforovich, 1994). The computational methodology applied to these peptides was largely with the view of determining very precise structural information about particular low-energy forms, usually to complement NMR and crystallographic

studies. These approaches are of necessity expensive in computational time and this may have led to the view that molecules such as peptides are 'too flexible' to model, focussing attention upon constrained analogue approaches.

Methodology, applicable to the conformational analysis of flexible peptide substrates, was developed using dipeptides as a starting point, because they are relatively simple, biologically relevant molecules. Preliminary attempts at modelling simple charged dipeptides using Tripos software (SYBYL) quickly identified Random Search as the most appropriate method to use (Grail and Payne, 2000; Marshall and Payne, 2001). Simulation of solvation was a critical element in the modelling procedure. For speed of computation, implicit distance-dependent solvation methods were chosen over explicit simulations. Random Searches of small charged peptides using explicit solvation were shown to produce comparable results but were much more time consuming. A dielectric constant of 80 was routinely used to simulate aqueous solution as low dielectric constants gave unrealistic minimum energy conformations with *cis* ω torsion angle values (Grail and Payne, 2000; Marshall and Payne, 2001). To summarise briefly, the procedure generates, by a stochastic searching procedure, a set of unique conformers that can be ranked according to their energy. Calculation of each conformer's energy is carried out using molecular mechanics where molecules are considered as being made up of a series of hard spheres (atoms) connected by springs (bonds). The internal strain-energy penalty of moving a molecule into a conformation compared with a theoretical 'energy perfect' state is calculated from a parameterised force field. This force field calculation uses atom and bond type definitions to calculate different energy contributions, e.g. bond stretch and bond bend. Summation of all these

individual energy terms results in a total energy that can be used to rank the conformers (equation ③).

③

$$\text{Energy} = \sum E_{str} + \sum E_{bend} + \sum E_{oop} + \sum E_{tors} + \sum E_{vdW} + [\ \sum E_{optional}\ ]$$

E_{str} = energy of bond stretch or compression, E_{bend} = energy of bending bond angles,

E_{oop} = out of plane bending of planar atoms, E_{tors} = torsional energy due to bond twisting,

E_{vdW} = energy due to van der Waals non-bonded interactions,

$E_{optional}$ = optional energy terms, e.g. electrostatics, constraints.

Energy ranking identifies an energy minimum, which allows calculation of a Boltzmann distribution that describes the contribution each conformer makes to the overall population (equation ④).

④

$$\text{Boltzmann Distribution} = e^{-\Delta E / RT}$$

R = gas constant = 1.98 cal K^{-1} mol^{-1} T = temperature K

ΔE = (energy of conformer – energy of minimum-energy conformer) kcal mol^{-1}

This approach produces a high-quality definition of conformer occurrence in solution compared with previous studies that were concerned with the detailed analysis of limited low energy forms.

[The computer-based conformational analysis methodology developed for dipeptides is described in papers (i), (ii) and (iii).]

Analysis of conformers produced by Random Search

Random Search places each unique conformation it finds into a database and presents the results as a molecular spreadsheet (MSS). The individual energy values

and the number of times each conformer has been found (count) are automatically reported. Torsion angle and inter-atomic distance measurements were carried out and the values entered into the MSS.

Analysis of these unique conformers involved sorting and categorising each one on the basis of its backbone torsion angle (ψ, ω, ϕ) values. An initial division was made between conformers with *cis* or *trans* ω bonds. To analyse the ψ and ϕ angles their 360 ° range was divided into twelve 30 ° sectors. For dipeptide ψ angles, these are labelled A1-A12, and for ϕ, B1-B12 (Fig. 2). A more detailed analysis can be achieved using 10 ° increments, the torsion angle range being divided into 36 equal sectors. Conformers were categorised, according to their backbone torsion angle values, and the individual percentage contributions summed to give an accumulated percentage contribution (% contribution) for each ψ/ϕ sector combination. To manually sort and categorise the large number of conformers produced by each Random Search would be time-consuming and laborious. This problem was overcome by using dedicated programs, written in SYBYL programming language (SPL), that sorted the conformers based upon their ψ-ϕ torsion angle values and summed the % contribution in each ψ/ϕ sector combination. The program outputs the results to a file in the format required to import them into a graphical package (SigmaPlot). These data are best displayed as three-dimensional graphical representations based upon a modification of the conventional Ramachandran plot. For dipeptides, plotting ψ (Tor2) against ϕ (Tor4) against percentage contribution results in a 3D-pseudo-Ramachandran plot (3DPR) (Grail and Payne, 2000; Payne *et al.*, 2000a; Payne *et al.*, 2000b). 3DPR can be plotted for individual peptides or, to help highlight conformational similarities between groups of peptides, data for several peptides can be plotted on a single graph. This

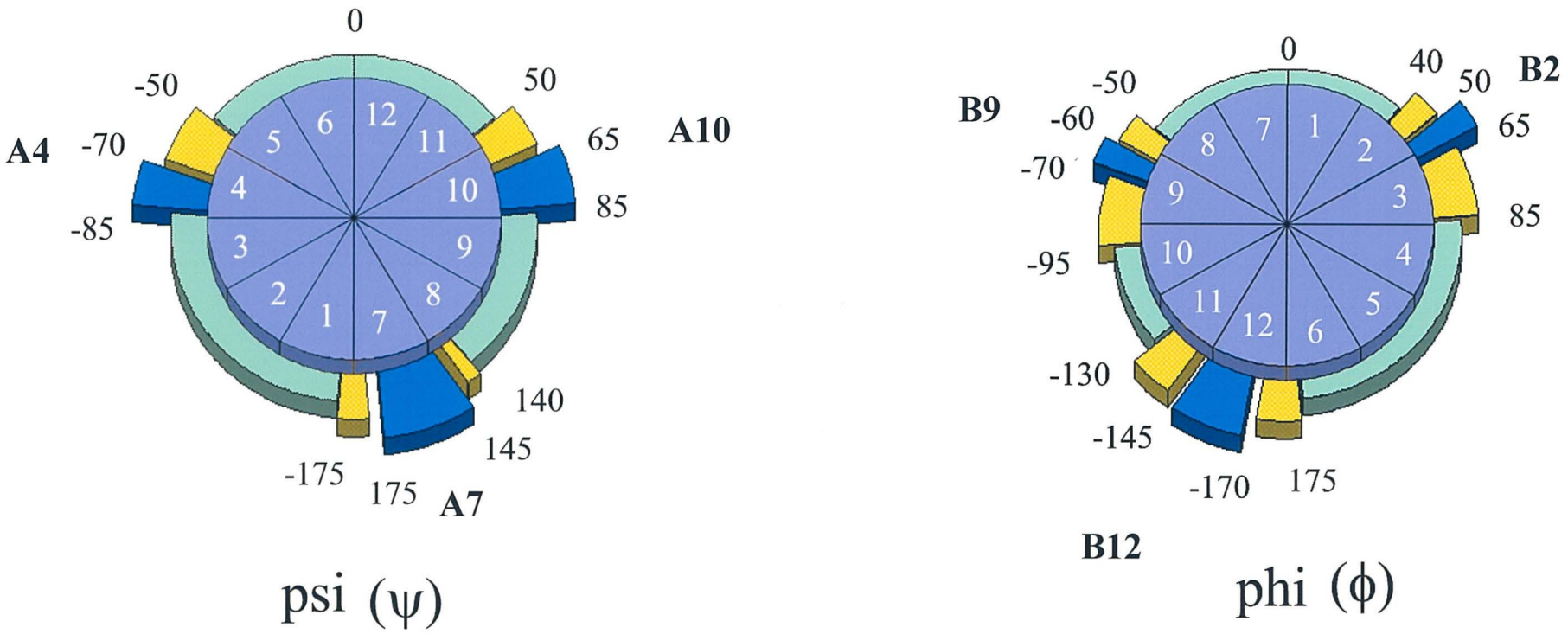

Figure 2. Torsion angle sectors for psi (ψ) and phi (ϕ) angles

Pie chart showing the sectors occupied by Tor2 psi (ψ) and Tor4 phi (ϕ) torsion angles in dipeptide conformers with *trans* ω bonds. Torsional space was divided into twelve 30 ° sectors (light blue) designated A1-A12 for ψ and B1-B12 for ϕ. Sectors are coloured to distinguish narrow (dark blue) torsion ranges excluding, or wider (gold) torsion ranges including, Gly and Pro residues; e.g., A7 sectors cover the range +145 ° to +175 ° for the dipeptides excluding Gly and Pro residues and +140 ° to –175 ° for all dipeptides.

innovative method of visualising the torsion angle distribution, with emphasis placed upon the contribution of particular backbone forms, plays a fundamental part in the definition of the conformational features of peptide transporter MRTs.

[The use of 3D-Ramachandran plots to visualise the conformational profiles of peptides was first described in (i) and is an invaluable tool in the conformational analysis of peptides (ii, iv, v). The outcome of Random Searches for a set of dipeptides are reported in paper (ii).]

Peptides adopt preferred backbone conformations in solution

The conformational analysis of individual dipeptides, the aggregation of these data and visualization in 3DPR plots, highlight the predominant conformations adopted by the backbone in solution and add greatly to the information derived by biophysical methods. Each of the 400 possible dipeptides has a conformer profile, with particular features shared with all other dipeptides as well as conformations that are unique to it or a limited group of sequence-related peptides. The distribution of *trans* ω conformers, when weighted according to their contribution to the conformer pool, was restricted to only nine preferred backbone conformations. These nine predominant ψ/ϕ combinations have ψ angle ranges of A7 (+140 ° to −175 °), A4 (−50 ° to −85 °) and A10 (+50 ° to +85 °), which are combined with ϕ angle ranges of B9 (−50 ° to −95 °), B12 (−130 ° to +175 °) and B2 (+40 ° to +85 °) (Fig. 2) (Grail and Payne, 2000). The spread of backbone conformations within dipeptides means that these torsion angle ranges do not exactly match the 30 ° sector definitions, but for simplicity they retain the original nomenclature. Individual dipeptides, although having the same main conformational types, vary in the distribution of conformers between these types. For example AlaAla has conformers

that are tightly distributed within the nine main ψ/ϕ torsional sectors, whilst LeuLeu, with branched aliphatic side chains, has conformers that are predominantly A4 and tend to spread across the main ψ/ϕ torsion angle ranges (Grail and Payne, 2000). The differences in conformer distribution for even quite similar dipeptides, makes analysis of their conformational similarities difficult. Considering such dipeptides as a group and aggregating their percentage contribution prior to visualisation in 3DPR plots highlights the main backbone forms and the shared conformations. Tripeptides and higher oligopeptides also adopt preferred conformations in aqueous solution that are distinct from those seen in regular protein secondary structure, e.g. α-helices, β-sheet (Marshall *et al.*, 2001). The predominant initial ψ_{i-1} (Tor2) values are in the ranges +150 ° to ±180 °, +60 ° to +90 ° and −60 ° to −90 °. These sectors are combined with preferred ϕ_i (Tor4) sectors of −150 ° to ±180 °, −60 ° to −90 ° and +30 ° to +60 °. The finding that peptides exist with a limited number of backbone conformations may seem unexpected in light of the perceived flexibility of peptides reported in the literature (Cornell *et al.*, 1997; Marshall *et al.*, 1993; Nikiforovich, 1994).

Definition of the nine main dipeptide backbone forms greatly assists the search for peptide transporter MRT conformations, as well as those for many other peptide-recognising proteins. The predominance of particular conformations may be attributed to specific stabilising features that influence the energy of the conformation. Understanding the energetics of these stabilised conformational forms is an interesting area of study that awaits further investigation and may add another aspect to the rational design of compounds - tailoring of conformational propensity (Payne *et al.*, 2001).

[The conformational analysis of di- and tripeptides is detailed in papers (ii) and (iv).]

V. DEFINING PEPTIDE TRANSPORTER MRTs

Backbone conformations recognised by dipeptide transporters

The percentage contributions for each of the nine main backbone forms in a set of dipeptide substrates were calculated. The proportions of conformers in these sets of conformational types were related to the substrate specificities determined experimentally for Dpp and Tpp. For Dpp, a correlation was found with the combined proportion of A7B9 and A7B12 backbone conformations. The results from assays of Tpp activity correlated well with a different combination of backbone conformers, namely A4(B9+B12) and A10(B9+B12). The distribution of conformers between these recognised forms in dipeptides makes them good substrates for both transporters (Payne *et al.*, 2000b).

Dipeptide MRTs

Initial studies identified the distinct dipeptide backbone conformers recognised by peptide transporters (Payne *et al.*, 2000b). To further define the MRT for dipeptide substrates the following seven structural features were considered:

(i) N-terminal α-amino and C-terminal α-carboxyl groups

The presence of charged N-terminal α-amino and C-terminal carboxylate groups are vital for optimal recognition by Dpp and Tpp, and are manifest in a distinctive charge distribution around the dipeptide molecule. The N- and C-termini are also groups with H-bonding potential. Both the charge and H-bonding features of the N- and C-

termini have a vector element, the correct alignment of which is essential for the consolidation of the initial binding complex.

(ii) Backbone torsion angles ψ, ω and ϕ

The majority of protein-derived dipeptides adopt conformers in solution that fall within the defined ψ/ω/ϕ regions recognised by Dpp or Tpp, making them good substrates for these transporters. Dipeptide conformers that exist outside of these optimal regions are poorly recognised; an example is GlyGly, which has a high proportion of unrecognised *cis* ω conformers (Brandsch *et al.*, 1998; Brandsch *et al.*, 1999; Payne *et al.*, 2001) as well as nominal Dpp conformers at the extreme edge of the MRT regions. Experiments show GlyGly to be a very poor Dpp substrate (Smith *et al.* 1999).

(iii) Stereochemistry at the α-carbon atoms

Peptide transporters have evolved substrate specificity that reflects the LL-stereochemistry of the dipeptide pool derived from protein hydrolysis. Peptides with mixed stereochemistry, i.e., LD or DL, are recognised poorly, whereas DD-dipeptides are not substrates for the transporters.

(iv) Distance between the terminal amino and carboxylate groups (N-C)

The distinct backbone torsion angle combinations recognised by Dpp and Tpp result in distance geometries with characteristic N-C distances. The ψ/ϕ combinations recognised by Dpp (A7(B9 + B12)) have N-C distances around 5.4 Å and 6.2 Å, respectively; in contrast, Tpp substrates (A4(B9 + B12), A10(B9 + B12)) are shorter, with N-C distances around 4.7 Å and 5.2 Å.

(v) Chi (χ) torsion angles of the side chains

The side chain binding pockets of peptide transporters generally accept the *t*, g^+ and g^- χ torsion angle orientations seen in dipeptides. However, for some amino acid side chains particular χ angle values may compromise other important recognition features, e.g. the charged amino terminus.

(vi) Hydrogen bond donor and acceptor properties of the peptide bond

Normal *trans* peptide bonds form optimal H-bonding interactions with the transporters. In contrast *cis* ω bonds are not recognised. Peptide bond modifications, e.g., *N*-methylation, remove the hydrogen bonding potential of the amide nitrogen resulting in poor recognition. Similar effects are seen with peptides that have distorted ω torsions i.e. outside $\pm 180 ° \pm 5 °$, where the optimal vector for interaction becomes progressively diminished. Losses in H-bonding potential such as these reduce the proportion of MRT conformers and so result in a lowered activity.

(vii) Charge distribution around the N- and C-termini

Dipeptide conformers have a characteristic charge distribution around their N- and C-termini, which in MRT backbone forms is optimal for charge-charge interactions with cognate side chain groups in the protein. Alterations in this charge distribution that adversely affect these consolidating interactions will reduce activity (see *(v)* above).

Tripeptide MRTs

Dpp and Tpp also transport tripeptides to a limited extent, which implies a degree of conformational overlap between the di- and tripeptide substrate pools (Smith *et al.*, 1999). Analysis of the N-C distance distribution for a set of dipeptides defined the

distances accommodated by Dpp and Tpp. A similar analysis of tripeptides identified a subset of "folded" forms with the required backbone torsion angles and N-C distances for recognition by Dpp or Tpp. The proportion of these "folded" forms in a set of tripeptides related well with their experimentally determined activity for Dpp or Tpp (Payne *et al.*, 2000b).

[The MRTs for di- and tripeptide transporters are outlined in paper (i) and described in detail in paper (iii).]

Oligopeptide transporters

Opp binds and transports oligopeptides, and to a much lesser extent dipeptides. Conformational analysis of a set of oligopeptides (3-5 residues) defined the N-terminal backbone torsions and N-C distances adopted by these substrates. An A7B9 ψ/ϕ combination predominated, with A7B12 also present but in lesser amounts. Determining the N-C distance distribution identified two forms within the conformer population, folded structures having N-C distances around 5.5 Å and an extended set of conformers with N-C >6.5 Å. Ligand-binding results for a set of tripeptides with OppA correlated with the percentage of conformer for each peptide present as A7B9 with an N-C >6.5 Å (Marshall *et al.*, 2001; Payne *et al.*, 2000b).

[The MRT for oligopeptide transporters is described in papers (i) and (iv).]

Kinetically-driven ligand binding

Ligand binding is generally viewed as being thermodynamically-driven; high affinity substrates form more stable protein-ligand complexes compared with low affinity substrates. These complexes are seen as being differentially stabilised by consolidating interactions such as H-bonds or rearrangement of water in binding

pockets (Tame *et al.*, 1996). The results from these studies with peptide-binding proteins imply an alternative, broader viewpoint (Grail and Payne, 2000; Marshall *et al.*, 2001; Payne *et al.*, 2000a; Payne *et al.*, 2000b). Peptide transporters optimally recognise and transport a wide range of peptides using a recognition mechanism that overcomes the differences in amino acid sequence and concentrates upon the commonality of backbone conformation. Crystal structure data, along with our studies, have shown this to be the case; salt-bridge and H-bond interactions are made with the peptide backbone and the amino acid side chains sit in large, hydrated pockets (Dunten and Mowbray, 1995; Marshall *et al.*, 2001; Payne *et al.*, 2000a; Tame *et al.*, 1995). The correlations between the proportion of MRT conformers in a peptide's conformer population and activity for each peptide transporter (Dpp, Tpp, Opp) confirm that these systems are primarily kinetically-driven, and that the peptide-specific differences in affinity are attributable to MRT occupancy. Trying to relate thermodynamic elements to these differences in activity has shown DppA and OppA to be heavily enthalpy/entropy compensated (Payne *et al.*, 2000a). Kinetically-driven reactions, such as ligand binding by peptide transporters, respond positively to increases in the proportion of active conformer in the Boltzmann-weighted conformational population. Occurrence of flexible substrates in restricted conformational types has an effect analogous to constraining a molecule by covalent bonding, and minimises the loss of conformational entropy upon binding of the ligand. It is interesting that enzymes such as carbonic anhydrase and catalase, whose substrates have very limited conformational freedom, have extremely high turnover numbers. It is likely that both thermodynamic and kinetic elements combine to make up the overall activity measured in many biological systems and approaches to

evaluate the relative contribution from each will be required to understand them fully.

Structure-activity relationships of peptide transporters

These studies have brought together biochemical, biophysical and computational techniques to understand the structure-activity relationships of peptide transporters. Conformational analysis of di- and tripeptides has shown that they adopt a limited set of backbone conformations in solution, amongst which particular forms have been identified that are recognised and bound by peptide transporters. The steric and electronic properties of peptides are important features that influence the conformational distribution in solution, as well as being involved in consolidating protein-peptide complexes. The complement of peptide transporters has evolved to recognise distinct peptide backbone forms and so fully exploit the substrate pool. Identification and quantification of MRT conformations for the bacterial peptide transport systems has shown them to be kinetically-driven. The definition of shared structural MRT features for di- and tripeptides endorses the principle that peptide transporters recognise the same backbone forms regardless of their protein architecture or method of energisation.

VI. CONFORMATIONAL ANALYSIS OF TRANSPEPTIDASE SUBSTRATES

Transpeptidases catalyse the joining of nascent cell-wall peptides in peptidoglycan. These important enzymes are targets for β-lactam antibiotics, which are potent suicide substrates. Peptidoglycan structure and penicillin-binding proteins have been extensively researched to understand enzymic recognition of cross-linking peptides, and the action of β-lactam antibiotics as substrate mimics (Frau *et al.*, 1998; Frau and

Price, 1996; Kelly *et al.*, 1985; Labischinski *et al.*, 1985; Tipper and Strominger, 1965; Virudachalam and Rao, 1977). As an experimental system, cell-wall peptides offer the opportunity for conformational analysis of a peptide substrate with unique structural features that distinguish them from the products of protein digestion, as well as being an important target for antimicrobial agents.

Peptidoglycan structure

Peptidoglycan is a main structural element of both Gram-positive and Gram-negative cell walls. It is a complex heteropolymer, strengthened by cross-linking individual polysaccharide chains with peptide bridges into a sack-like mesh (Park, 1996). The polysaccharide chains consist of alternating *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM) subunits. Peptide bridges are joined to NAM residues at intervals along the polysaccharide chain and are made up from L-Ala, D-Glu, L-Lys and D-Ala with some limited species-specific modifications, such as changing the L-Lys to *meso*-diaminopimelic acid or L-ornithine, or the D-Glu to D-Gln. To link separate NAG-NAM chains, the ε-amino nitrogen of L-Lys from a peptide on one chain is covalently bonded directly, or through a pentaglycine bridge, to the penultimate D-Ala of a peptide on an adjacent chain, with concomitant release of the terminal D-Ala residue.

Conformational analysis

Model cell-wall peptides.

The work of DeCoen established di-acyl-L-Lys-D-Ala-D-Ala (Ac_2KDADA) as the best model for the cross-linking peptides in peptidoglycan (De Coen *et al.*, 1981; Frau and Price, 1996). Applying conformational analysis methodology developed for

small flexible peptides to this modified tripeptide, produced results that were similar to, but distinct from, those obtained for di- and tripeptides (Grail and Payne, 2000; Marshall *et al.*, 2001). 3DPR and 3D-Ramachandran (3DR) plots were obtained for pairs of ψ/ϕ torsion angles, starting with Tor8/Tor6 and ending with Tor4/Tor6 (Fig. 3). As seen for other peptides (Grail and Payne, 2000; Marshall *et al.*, 2001), the conformer profiles obtained were restricted, with particular ψ/ϕ combinations being preferred. There are six main backbone conformations formed by the combination of three different carboxyl group orientations (determined by Tor8) with two different Tor6 angle ranges. The main Tor8 torsion angle ranges are, 50 ° to 70 ° (D3), 140 ° to 170 ° (D6) and −50 ° to −70 ° (D9) combined with a Tor6 of either −50 ° to −80 ° (C4) or 30 ° to 50 ° (C11). Tor4 torsion angle values are largely confined to 30 ° to 70 ° (B2) and −40 ° to −70 ° (B8) that combine with Tor6 in two particular combinations: C11B2, C4B8 (Grail and Payne, 2002). The combination of each Tor6 angle range with only one particular Tor4 angle range highlights the fact that seemingly flexible substrates can be highly conformationally constrained. For maximum efficiency, transpeptidase might be expected to recognise as many conformational forms as possible, their selection being influenced by shared recognition features and compatibility with the catalytic mechanism. The two predominant Tor6/Tor4 combinations are probably mutually exclusive in this respect, because the different L-Lys side chain orientations make alignment of the scissile bond with the catalytic centre for both combinations difficult (Grail and Payne, 2002). The natural selection of MRT features will also be influenced by the presence of co-evolving inhibitor strategies. As well as providing a driving force for transpeptidase evolution, the substrate conformer profile will also drive the natural

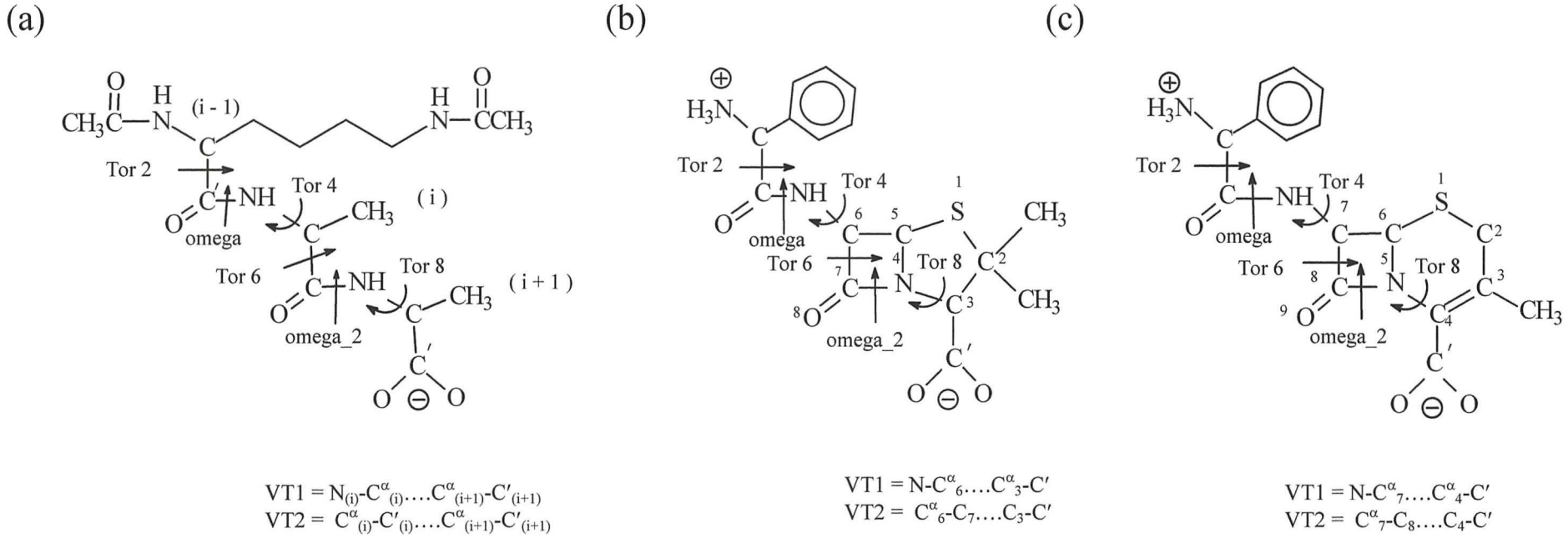

Figure 3. Backbone and virtual torsion angles of model cell-wall peptides and β-lactam antibiotics

Structures of (a) di-acyl-L-Lys-D-Ala-D-Ala, (b) ampicillin and (c) cephalexin are presented with the torsion angles used for conformational analysis numbered and indicated by arrows. For clarity, non-essential hydrogen atoms are not shown, but the correct valency is maintained throughout.

selection of antibiotics based either upon substrate mimicry (β-lactams) or competition (vancomycin).

[The conformational analysis of model cell wall peptides is described in paper (vii).]

β-lactam antibiotics

Conformational analysis of selected β-lactam antibiotics and visual display in 3DPR plots shows that the penicillins adopt two main forms at Tor8, axial with a torsion angle value of 135 ° and equatorial with a value near 90 °. Δ^3-cephalosporins are more constrained and only adopt a single Tor8 form with a value of ~12 °. Tor7 and Tor6 form part of the lactam ring and are very constrained having values of ~160 ° and ~ −110 °, respectively, making their direct comparison with peptide backbone conformations difficult (Fig. 3).

Virtual torsion angles

An alternative comparison between peptide substrates and the constrained β-lactam structure is possible if virtual torsion angles (VT) are used (Gupta *et al.*, 2002). These VT span the distorted ω found in β-lactams and define the relative spatial orientation of the carboxyl group without necessarily using contiguous backbone atoms as in traditional torsion angles ψ, ϕ and ω (Fig. 3). Measurement of VT1 and VT2 for the model cell-wall peptide, and display in a three-dimensional virtual torsion (3DVT) plot defines the areas characteristic of the main Tor6/Tor8 torsion sectors. Similar VTs were measured for β-lactams and compared with the peptide substrate using a contour plot. This contour plot highlights the structural similarity

between these suicide substrates and the cross-linking peptide; it also facilitates superposition of related conformers.

[The virtual torsion angle combinations of dipeptide MRT conformations are described in paper (vi) and are used to compare model cell wall peptides to β-lactam antibiotics in paper (vii).]

MRT for transpeptidase

3DPR and 3DR plots allow initial comparison of the principal β-lactam forms with the main Ac_2KDADA backbone conformations and indicate a common recognition mechanism involving a Tor8 around D3 combined with a Tor6 of C4. This inferred similarity is endorsed by inspection of VT contour plots where the predominant β-lactam forms all fall on a line representing a Tor6 of C4 and cluster around a region representing D3C4 VT1VT2 combinations (Grail and Payne, 2002). Rigid 6-atom fits of representative β-lactam conformational forms onto D3C4 conformers of Ac_2KDADA using VT1 and VT2 atoms, in combination with atoms that form important interactions with transpeptidase, produce good superpositions over most of the lactam nucleus. However, significant displacement of the carboxyl group is seen in those β-lactams with the poorest antibacterial activities, implying that the correct alignment of this charged group is important for recognition and productive binding. Aligned molecular graphics of representative conformers can also be used to visualise and identify common structural features within substrates (Grail and Payne, 2002).

The constrained nature of the lactam ring, and the bulky or aromatic N-terminal side chain causes β-lactam conformers to adopt a single, predominant Tor4 around 165 °. The different Tor4 seen between the two substrates suggests that this

torsion is not critical for recognition. Furthermore, an MRT for initial recognition, consolidation of binding and acylation of transpeptidase, is largely defined by a D3 carboxyl orientation having a Tor6 of C4 and by the long/bulky side chain being orientated "above" the peptide or pseudopeptide backbone. The precise mechanism of binding of both the peptide substrate and β-lactam antibiotics to transpeptidase is still largely unknown (Kelly *et al.*, 1985; Lee *et al.*, 2001; Massova *et al.*, 1999). Studies on the active-site serine PBP have shown that the active-site topography is well conserved and brings about transpeptidation using a two-stage acylation/deacylation reaction mechanism (Goffin and Ghuysen, 1998; Knox *et al.*, 1996; Lee *et al.*, 2001). Acylation is brought about by withdrawal of a proton from Ser62 γO producing an oxyanion, which attacks the peptide-bond carbonyl group. The proton is donated back to the peptide-bond nitrogen, resulting in bond breakage and formation of an ester link to Ser62. Deacylation of the acyl-enzyme complex requires activation, by proton removal, of an incoming acceptor (amino group or water), which then hydrolyses the ester linkage, the proton subsequently being donated back to the γO to reform the hydroxyl of Ser62 (Goffin and Ghuysen, 1998). The positions of the CO-NH atoms of the peptide bond linking the L-Lys residue to D-Ala-D-Ala seem to be influential in optimising the direction of approach for hydrolysis of the acyl-enzyme intermediate (Lee *et al.*, 2001; Massova and Mobashery, 1998). The MRT for a substrate could, therefore, be extended for optimal deacylation of the acyl enzyme complex by including a Tor4 orientation of B8.

Early studies focussed upon the chemical reactivity of β-lactams to try to understand the effectiveness of different β-lactam antibiotics, but subsequent studies have shown that they are in fact poor acylating reagents and certainly no better than

peptide substrates (Page, 1999). These results imply that the MRT occupancy and differences in how bound conformers are processed by the catalytic mechanism could be important factors in understanding the action and varied efficacies of different β-lactam antibiotics.

Inhibition of transpeptidase by β-lactam antibiotics

β-lactam antibiotics have evolved to be suicide substrates for transpeptidase through formation of stable acyl-enzyme intermediates. They bind well and are able to take part in the acylation reaction, as would be expected from them possessing the required MRT features; structural similarity over Tor8, Tor6 and the aminoacyl side chain positioned on the 'convex' side of the molecule. Their non-competitive inhibition of transpeptidase is considered to be due to the inability of the catalytic apparatus to deacylate the enzyme-antibiotic complexes; however, as of yet it is unclear which features of β-lactams are responsible for this inhibition. X-ray structures of DD-peptidase show that cell-wall peptides and β-lactam antibiotics form similar interactions with the B3 β-strand via the carboxyl terminus and the carbonyl group of the C-terminal peptide bond. H-bonding of the CO-NH of the penultimate peptide bond of the enzyme-linked cell wall peptide also occurs within the active site, providing stabilising interactions that may be critical for effective deacylation and which are lost by β-lactams due to the alternative Tor4 (Knox *et al.*, 1996; Lee *et al.*, 2001). Interestingly β-lactamases, which have evolved from PBP, are able to effectively deacylate the acyl-enzyme intermediate (Goffin and Ghuysen, 1998; Massova and Mobashery, 1998). This has been achieved by modification of the active-site topography. The ω loop at the bottom of the substrate-binding site in DD-peptidase has been twisted and a catalytic base introduced in the class B β-

lactamases. Tilting the B3 β-strand has also optimised the interaction between the enzyme and the specific Tor4 seen in β-lactams.

[The MRT for transpeptidase and its inhibition by β-lactam antibiotics are discussed in paper (vii)]

Glycopeptide antibiotics

The glycopeptide antibiotics, e.g. vancomycin and ristocetin, have evolved to bind tightly to cross-linking peptides and the peptidoglycan precursor, lipid II (undecaprenyl-(muramyl-glucosaminyl)-pentapeptide), and cause inhibition by removing substrate from transglycosylase and transpeptidase reactions. The evolution of both vancomycin and transpeptidase will have been driven by the conformer profile of their common substrate, but transpeptidase may also act as a selective pressure upon the antibiotic by directing its conformer specificity. The effectiveness of vancomycin is maximised by not having to compete for transpeptidase MRT conformers, the sequestering action of vancomycin eventually outstripping the catalytic activity of transpeptidase. In a similar manner, peptide transport systems have evolved to exploit optimally the peptide substrate pool by having complementary systems that recognise distinct conformational forms (Payne *et al.*, 2000b). Williams and co-workers elucidated the NMR structure of two glycopeptide antibiotics, vancomycin and ristocetin, and also proposed a mechanism of ligand binding based upon recognition of a C-terminal D-Ala and consolidation of this initial complex by numerous H-bonds (Popieniek and Pratt, 1991; van Wageningen *et al.*, 1998; Williams *et al.*, 1983; Williamson *et al.*, 1984).

Modified cell-wall peptides

The conformational profile of Ac_2KDADA is similar to but distinct from the profiles produced by modified cell-wall peptides such as the depsipeptide, diacyl-L-Lys-D-Ala-D-Lactate (Ac_2KDADLac), and diacyl-L-Lys-D-Ala-D-Ser (Ac_2KDADS) found in vancomycin-resistant strains (Grohs *et al.*, 2000; van Wageningen *et al.*, 1998). 3DPR analysis of these cell-wall peptides showed that they all retained the Tor8 (D3) MRT backbone forms recognised by transpeptidase, but the vancomycin-resistant peptides had a much smaller proportion of conformers with a Tor8 (D6) carboxyl orientation. The Tor4/Tor6 pairings seen in Ac_2KDADA are mirrored in both Ac_2KDADLac and Ac_2KDADS, providing two distinct forms, C4B8 and C11B2, in terms of recognition. Since C4B8 combined with a D3 carboxyl orientation is recognised by transpeptidase, the D6C11B2 forms are favoured candidates for recognition by vancomycin because they assist vancomycin binding through minimised steric hindrance from transpeptidase (Grail & Payne 2002).

Peptide binding by vancomycin

Crystal structures of vancomycin are in agreement with the NMR data, and analysis of co-crystallised Ac_2KDADA shows that its final bound conformation is different to the predominant conformational types seen in 3DPR plots (Grail and Payne, 2002). The bound ligand has a D6, *trans* omega_2 C-terminal conformation combined with Tor6 and Tor4 angle values of –160 ° and 145 °, respectively; this can be identified by conformational analysis but it has a high energy in solution and accounts for less than 1% of the conformer population. Kinetic studies on the interaction of vancomycin with cell-wall peptides have shown it to be a two-stage process; initial recognition being the fast docking of the peptide's C-terminus followed by a slower

consolidation of the complex (Kurz *et al.*, 1996; Popieniek and Pratt, 1991). It seems likely that the initial interaction with vancomycin could be with a predominant conformer that has the required carboxyl orientation with induced, torsion angle changes optimising stabilisation of the final complex.

MRT for vancomycin

D6C11B2 backbone conformers are distinct from the form recognised by transpeptidase and have a C-terminal portion in the required orientation for initial binding. The remaining backbone is suited for induced conformational changes driven by a need to decrease steric interaction between the penultimate methyl group and e.g. a chlorine atom in vancomycin, and the formation of stabilising hydrophobic interactions. The alternative C4B8 backbone conformation is less suited to these induced changes, and although D6C4B8 conformers may initiate interactions, the position of the L-Lys side chain "above" the backbone would interfere with the smooth "zippering" required to produce the stable complex.

Studies have tried to equate the >1000 fold difference in affinity between the normal peptide substrate and vancomycin-resistant depsipeptide substrate to the loss of one H-bond. However, the ~20 fold reduction in affinity consequent upon replacing Ala with Ser is attributed to the loss of stabilising hydrophobic interactions. Modelling both these modified substrates, as blocked tripeptide analogues, has shown alterations in their conformational profiles such that they can still be recognised by transpeptidase (D3C4B8) but the recognition features required by the glycopeptide antibiotic (D6C11B2) are diminished. The combination of these conformational changes, along with the loss of stabilising interactions, provides a more realistic basis for the large differences in affinity seen experimentally.

[The conformational profiles of modified cell-wall peptides are described in paper (vii) along with consideration of the MRT for vancomycin.]

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APPENDIX

PUBLICATIONS

i. **Payne, J. W., Grail, B. M. and Marshall, N. J. (2000)** Molecular recognition templates of peptides: Driving force for molecular evolution of peptide transporters. *Biochem. Biophys. Res. Commun.* **267**, 283-289.

ii. **Grail, B. M. and Payne, J. W. (2000)** Predominant torsional forms adopted by dipeptide conformers in solution: Parameters for molecular recognition. *J. Pept. Sci.* **6**, 186-199.

iii. **Payne, J. W., Grail, B. M., Gupta, S., Ladbury, J. E., Marshall, N. J., O'Brien, R. and Payne, G. M. (2000)** Structural basis for recognition of dipeptides by peptide transporters. *Arch. Biochem. Biophys.* **384**, 9-23.

iv. **Marshall, N. J., Grail, B. M. and Payne, J. W. (2001)** Predominant torsional forms adopted by oligopeptide conformers in solution: Parameters for molecular recognition. *J. Pept. Sci.* **7**, 175-189.

v. **Payne, J. W., Payne, G. M., Gupta, S., Marshall, N. J. and Grail, B. M. (2001)** Conformational limitations of glycylsarcosine as a prototypic substrate for peptide transporters. *Biochim. Biophys. Acta-Biomembr.* **1514**, 65-75.

vi. **Gupta, S., Grail, B. M. and Payne, J. W. (2002)** Presence of distinct virtual backbone torsion angles in dipeptide conformers. *Prot. Pept. Lett.* **9** 133-138.

vii. **Grail, B. M. and Payne, J. W. (2002)** Conformational analysis of bacterial cell wall peptides indicates how particular conformations have influenced the evolution of penicillin-binding proteins, β-lactam antibiotics and antibiotic resistance mechanisms. *J. Mol. Recog.* **15** 113-125.

The seven papers presented are the result of a body of work carried out in Prof. J.W. Payne's laboratory in Bangor. Work in this laboratory has endeavoured to characterise peptide transporters and understand how they recognise and bind peptide ligands. Biochemical and biophysical assays provide relative binding affinities for a range of peptides and peptide analogues but give no explanation of the differences seen between them or the differential activities between transporters. The application of computer modelling to these studies was seen as an advance that could help in understanding these issues. I was influential in bringing this technology to the laboratory and applying it to the study of substrate recognition by peptide transporters.

Papers i-iv

I carried out the majority of the conformational analysis of peptide transporter substrates detailed in these papers. These publications were possible largely because of the methodologies that I devised for searching flexible peptides and analysing the output from these searches. One long-term goal of the group has been to describe fully the structural and electronic features required for recognition by peptide transporters, the so-called molecular recognition template (MRT). The conformational searching procedure, data analysis methodology and graphical display of complex torsion angle data, that I developed, have allowed the common structural features of peptides to be determined. Integration of this computer-based analysis with biochemical and biophysical data resulted in the precise definition of MRTs for the bacterial peptide transporters Dpp, Tpp and Opp. These MRTs have shown Dpp, Tpp and Opp to be kinetically driven and that the substrate is influential

in the evolution of peptide transporters. Together they form a set of papers that make a significant contribution to the field of peptide transport in particular as well as molecular recognition in general.

Paper v

This paper focuses upon the peptidase-resistant pseudopeptide GlySar, which has become a standard substrate used to assay peptide transport. I used the methods I had devised using natural peptides to determine the conformational distribution of GlySar and related dipeptides. The results from these analyses were in good agreement with biological data and highlighted the limitations of GlySar as a general peptide transporter substrate. The extensive use of GlySar to measure mammalian intestinal transport means that this paper should prove of interest to the pharmaceutical industry. It also demonstrates the successful application of this methodology to pseudopeptides and helps to bridge the gap between bacterial and intestinal peptide transporter research.

Paper vi

This is a short, but important, publication that starts to address the problem of relating compounds with non-peptide backbones (pseudopeptides) to the MRTs defined for natural peptides. I wrote SPL macros that produced sets of conformers within the required backbone torsion sectors, which allowed the related VT1VT2 combinations to be determined. Again, the study relies heavily upon the conformational analysis and 3D graphical display techniques I had developed previously.

Paper vii

This substantial paper results from the application of the computer-based protocols I developed to an area of clinical importance. The results I obtained bring insight into the molecular recognition of substrates by transpeptidase and glycopeptide antibiotics, as well as the mechanism of inhibition of β-lactam antibiotics. The MRTs detailed in this paper will be of use in the rational design of new antibiotics and help in understanding antibiotic resistance mechanisms.

Assay of peptide transport and binding

These studies required the integration of computer modelling data with results from biological assays. The bacterial peptide transporters Dpp, Opp and Tpp were used as model systems and as part of these studies I have gained experience in assaying and purifying components of these systems. I have routinely purified *E. coli* peptide binding proteins from crude osmotic-shock fluid, using ion-exchange chromatography (FPLC). Purified binding proteins were characterised using SDS-PAGE, Western blotting and IEF. Western blotting was carried out using peptide binding protein specific polyclonal and monoclonal antisera, purified by Dr C. Schuster and myself as part of her Ph. D. studies. I also measured peptide binding to these purified proteins using [^{125}I] GlyTyr. In conjunction with other members of the laboratory I have assayed peptide uptake using fluorescamine and determined competition for particular peptide transporters using competition plate assays. These studies have given me a broad understanding of the biochemical tools used to study peptide transporters.

Computing

Initiation of this project in the laboratory required me to become proficient in UNIX and to administer initially one, and later three Silicon Graphics workstations. This involves a diverse range of skills including software and license installation, remote file system sharing as well as user account management. Teaching myself to use the SYBYL molecular modelling package took several months of intensive work and involved molecule sketching, molecular database construction, rendering and manipulation of imported crystal structure data and interfacing with the program at a level below the "user front-end" to facilitate data acquisition. Later in the project, I taught myself SYBYL programming language and wrote macros that proved vital in the full exploitation of the conformational searching methodology.

Modelling flexible peptides

When I started these studies, the published information on modelling small flexible peptides, particularly as zwitterionic molecules, was sparse. This required me to approach the problem from basic principles and develop new approaches for conformational searching and analysis. These methods have been used extensively by colleagues in the laboratory and are the basis of many recent publications. They are a significant advance in the field of computer modelling of peptides and other flexible molecules.

Conformational analysis and 3D graphical representation

Modelling of dipeptides provided a unique resource of conformational information that proved vital in understanding the important structural features required for

recognition by peptide transporters. Extracting and displaying, in an informative way, the information from this resource was not trivial. Methods for carrying out such an analysis were not available and so I wrote an SPL macro that would categorise the conformers according to their backbone torsions and aggregate their Boltzmann-weighted contributions. I have now written several improved, and extended, SPL macros that allow rapid calculation of conformer distribution and output data files that can be imported directly into graphical packages. The advantage this gives of displaying the data in novel 3D mesh plots has allowed the full exploitation of this approach and proved invaluable in communicating these results to the scientific community.