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INTERACTIONS OF VANCOMYCIN AND RISTOCETIN WITH PEPTIDES AS A MODEL FOR PROTEIN BINDING

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Abstract—The glycopeptide antibiotics vancomycin and ristocetin act by binding to peptides terminating in -D-Ala-D-Ala. Thermodynamic and kinetic parameters for the binding are evaluated and used in conjunction with previously determined stereochemical details to generate a complete picture of the binding interaction. A conformational change of the antibiotics is necessary to permit fast on-rates, and produces a hydrophobic pocket for the peptide carboxylate group. We discuss an unusual salt bridge and consider the origins of the high specificity of the antibiotics. The discussion is extended to macromolecule–substrate interactions. The importance of fast access to binding sites and complementarity of hydrogen bonding pairs is stressed.

Understanding the interactions between proteins and their substrates has long been a goal of chemists and biologists, but despite much effort, the picture is still far from clear. There are several reasons for this. The structure of free and bound protein is hard to define in adequate detail, as most proteins are too large to give high resolution X-ray or NMR data, while the number of interactions involved even in binding of small substrates is so large that few computational or physical techniques have been able to give anything but a general impression of the energetics involved, especially where conformational change is associated with the binding. There are even more problems with those proteins that are enzymes, because most substrates of interest are not bound long enough to be readily studied. In addition, the energetics are more complicated in that much of the "binding energy" is used to reduce the activation energy for the reaction catalysed, and therefore the observed binding energy is only what is left over after this utilisation.[1]

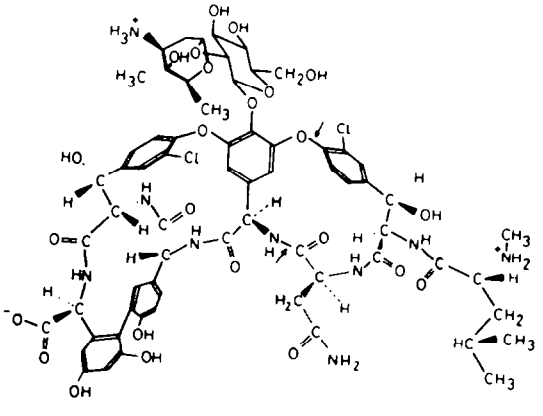
There are a number of features common to all binding proteins (in which we include enzymes) studies to date. They all have a more or less well-defined tertiary structure which alters on binding, often taking the form of a cleft shutting or a lid closing, to leave the substrate in a hydrophobic environment. The binding sites are specific for a small group of molecules, often showing remarkable discrimination between very similar compounds. They nearly all bind to substrates at diffusion-controlled rates, the differences in binding constant being due to differences in the off-rate. However, details of the structural features which lead to this behaviour are sparse. We present here work on the antibiotics vancomycin and ristocetin A, which also show such behaviour, and are small enough to be treated in sufficient detail that the rationale behind the binding can be seen in terms of individual bonds and atoms. The interactions found for these antibiotics have an obvious extension to proteins, for which we believe they can act as a model.

On a recent review,[2] we presented the structure and mode of action of the vancomycin group, and the reader is referred to it for further details. A short account of more recent work is given below.

Structure of the antibiotics

Following much chemical and spectroscopic work, the structure of vancomycin was inferred from the crystal structure of a derivative, CDP-I.[3] The conversion of vancomycin to CDP-I is effected by heating in water at pH 4.2 for 2 days at 80°, and it was assumed that this merely hydrolysed a primary amide, known to be present in vancomycin, to a free carboxylate. It now appears that this assumption was unjustified; an iso-aspartate residue found in CDP-I originates from intramolecular rearrangement of an asparagine residue in vancomycin,[4] and in the course of the rearrangement a chlorinated aromatic ring flips over by 180°.[5] The structure of vancomycin is now thought to be **1**, and that of CDP-I is **2**. As can be seen, vancomycin is a linear heptapeptide composed mainly of unusual aromatic amino acids oxidatively linked. It also contains glucose and an unusual amino sugar, vancosamine. The overall conformation shown in **1** is that assumed by CDP-I in the crystal structure, but NMR studies, particularly using the nuclear Overhauser effect (NOE),[5,6] indicate that this overall conformation is also the predominant one in solution.

Chemical and NMR studies had indicated that much of ristocetin A was similar to vancomycin.



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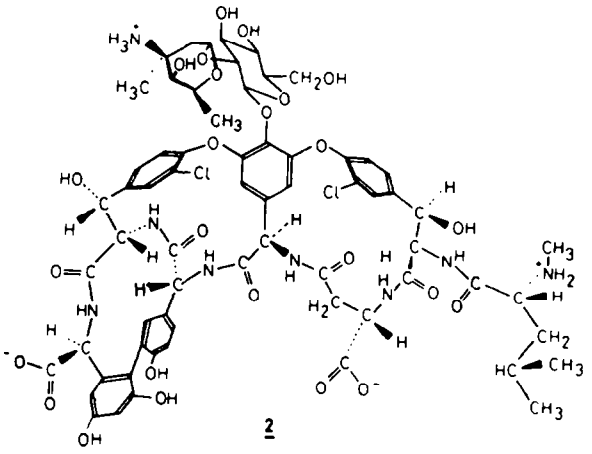

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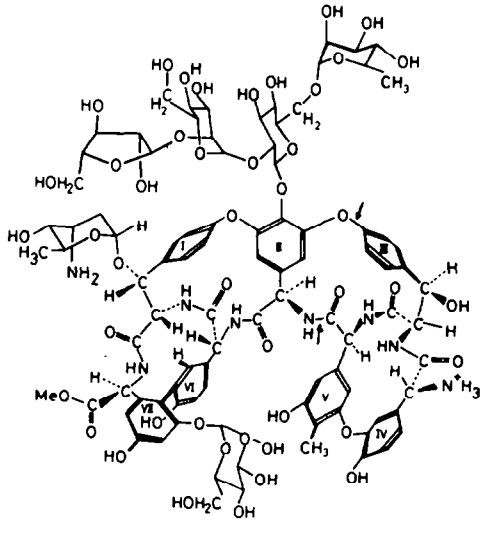
Following the crystal structure of CDP-I, it was possible to define the structure of ristocetin A *almost* entirely by NMR.[7,8] One stereochemical centre was wrongly assigned, as shown by Harris and Harris following careful chemical degradation,[9] and now confirmed by us by means of time-dependent NOE's.[10] The structure of ristocetin A is **3**. Ristocetin B lacks two sugars from the tetrasaccharide of ristocetin A, but is otherwise identical. All of our work was carried out on ristocetin A, while some of the previous binding studies were done using ristocetin B. For our purposes there is no difference between the two antibiotics, and hence both will be referred to as "ristocetin".

It can be seen from **3** that ristocetin is less flexible than vancomycin, since the N-methylleucine residue of vancomycin is replaced by an aromatic side-chain (ring IV), which is connected to ring V, further restricting its mobility. However, there is still considerable flexibility, as indicated by NOE experiments. Measurements of the rates of build-up of NOEs can give information on internuclear distances, since the NOE builds up at a rate proportional to r^{-6}.[5] If there is oscillatory motion, leading to a periodic alteration of internuclear distance about the equilibrium position, the NOE will build up very rapidly when the two protons are at their closest, and hence give rise to shorter apparent distances.[10] Calculation of distances from the protons α to rings V and IV to the ring protons *ortho* to them (Fig. 1) shows that the apparent distances are incompatible with normal bond lengths and angles. The deviation from expected distances shows that there is considerable rapid oscillation of rings IV and V, much larger in amplitude than that of the rings on the left-hand-side of both antibiotics.



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There are a number of other antibiotics in the vancomycin class,[2] all with similar structure. These are actinoidin, avoparcin,[11] A-35512B, and the newly characterised A-4696[12] and teichomycin A_2.[13]

Structures of the complexes

Vancomycin and ristocetin are active against gram-positive bacteria. They act by binding to mucopeptide precursors, which inhibits the biosynthesis of the cell wall. This weakens the cell wall and leads to eventual death by lysis. The structure of the mucopeptide varies according to species of bacterium,[14] but generally ends with -L-amino acid–D–Ala–D–Ala, the L-amino acid being lysine, ornithine or *meso* diamino–pimelic acid. UV studies[15-17] have shown that only the three C-terminal residues affect the binding constant, the two D-Ala residues being the most important. Substitution by an L residue prevents binding, and substitution of the Me group by a larger sidechain or hydrogen markedly weakens binding. The observed free energy of binding between vanocmycin and Ac_2–L–Lys–D–Ala–D–Ala ("tripeptide") is 8.5 kcal mol^{-1}; the complexes are thus both specific and strong.

The only source of information about the structure of the complexes comes from NMR, as the complexes have not yet been induced to crystallise in a form suitable for X-ray studies. (This is also true for free

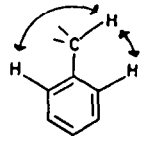

Fig. 1. Internuclear distances used to estimate extent of ring oscillation.

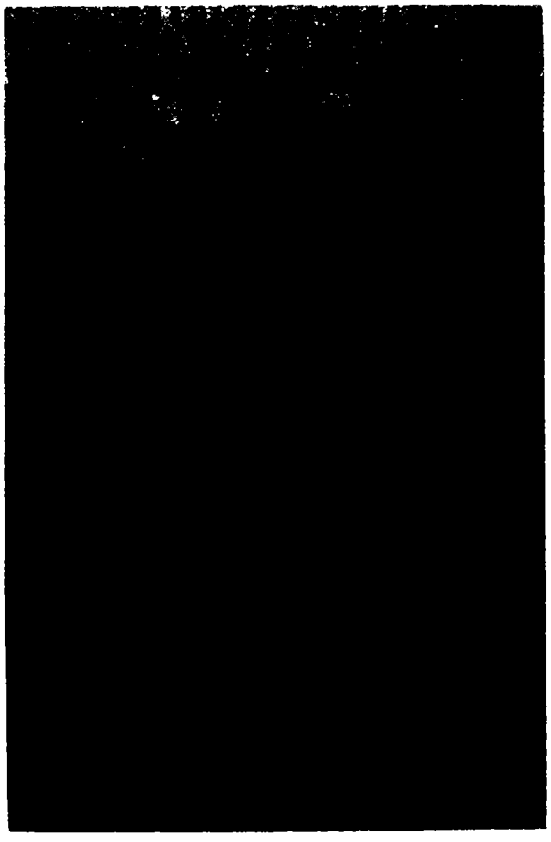

Fig. 2. Space-filling model of the ristocetin –Ac–D–Ala–D–Ala complex, exploded view. Hydrogen atoms with hooks are good hydrogen bond donors. Dashed lines indicate amide hydrogen bonds formed in the complex; dotted-and-dashed lines indicate bonds between the peptide carboxylate and amide NH groups on the antibiotic. Groups marked are: A, N-terminal alanine methyl; B, C-terminal alanine methyl; C, peptide carboxylate; D, tetrasaccharide R_2 (only glucose is included here, for clarity); E, ristocetin N-terminal amino group; F, ring IV; G, mannose R_1.

ristocetin and a number of its derivatives.) It has however been possible to get a good deal of information from NMR, especially from the NOE, including much information about the dynamics of the complexes which would be unobtainable from crystal studies. Examination of a space-filling model of ristocetin shows that there is a ready-made binding site for tripeptide (Fig. 2). This consists of three amide NH groups very close together, suitable for binding to a carboxylate ion, two other amide groups for further hydrogen bonding, and suitable hydrophobic regions corresponding to the hydrophobic parts of the peptide. In addition, there is a primary amino group, which would be protonated at neutral pH, close enough to the carboxylate binding site to interact with the carboxylate ion. NMR studies[18,10] have shown that this is indeed where the peptide binds. Measurements of internuclear distances by time-dependent NOE's has shown that the alanine Me groups sit very neatly in Me-shaped holes, removing the Me group from contact with water in most directions. The hydrogen bonds can be made into the ideal geometry without straining any bonds or pushing atoms too close together, and this "ideal" structure is compatible with all NMR data. The amino group is about 5 Å from the carboxylate and pointing in the wrong direction to form a salt bridge; some interaction is however still possible, as discussed below.

There is a further refinement of the structure of the complex, obtained from NOE's.[10] This is that ring IV folds in over the carboxylate ion, thus making one wall of a "hydrophobic pocket", the other side being composed of rings II and III and the C-terminal Ala Me. This pocket fits neatly round the carboxylate and excludes water from it. Formation of the pocket also locks ring IV into a more rigid conformation. The complex is shown in Fig. 3.

Vancomycin has a less obvious binding site, as two of the amide NH groups and the secondary amine are too far away from their corresponding positions in ristocetin to contribute to the binding interaction. However, we have shown[19,10] that vancomycin undergoes a large conformational change on binding, of which the most significant part is a series of rotations bringing the two NH groups into the same arrangement as those in ristocetin. The leucine sidechain, which flops "down" both in the crystal and in solution, is brought "up" and again makes one side of a hydrophobic pocket. The amino group, which was originally about 9 Å from the carboxylate binding site, is now adjacent to it and can partake in a somewhat bent salt bridge (Fig. 4). The leucine sidechain again is much more rigid in the complex than it was in the absence of peptide. These details make the carboxylate pockets in vancomycin and ristocetin very similar; the rest of the binding site is also similar, the biggest difference being the presence of a chlorine atom in vancomycin. It should be emphasized that in the common parts of the vancomycin and ristocetin structures which lie to the left of the arrows in **1** and **3**, the conformation postulated for the complexes is essentially that found in the X-ray structure of the corresponding portion of CDP-I.

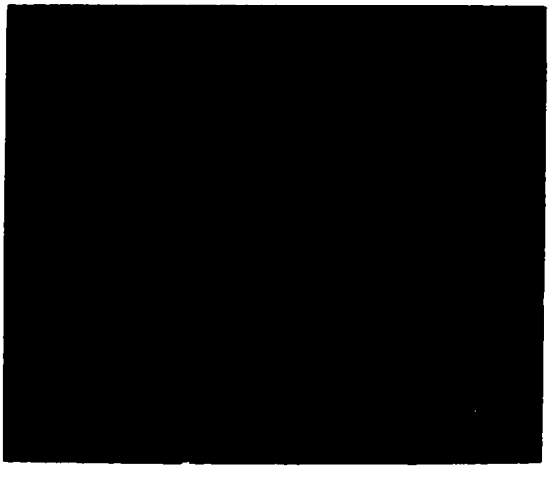

Fig. 3. Space-filling model of the ristocetin –Ac–D–Ala–D–Ala complex. Peptide protons are hatched. The model is viewed from above, and has been rotated clockwise about 60° relative to Fig. 2. Key as for Fig. 2. Note the folding in of ring IV, and the close fit of both alanine methyls into hydrophobic sites.

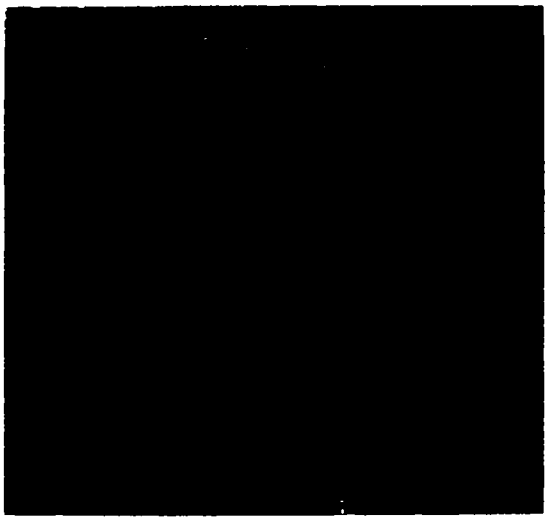

Fig. 4. Space-filling model of the vancomycin –Ac–D–Ala–D–Ala complex. Peptide protons are hatched. Viewed from above, and rotated about 30° anticlockwise from the corresponding position of Fig. 3. A, B, C, as Fig. 2; H, protonated amino group; J, leucine sidechain; K, front face chlorine atom; L, sugars. Note the "bent salt bridge" C to H.

RESULTS

In order to understand more fully both the kinetics and the thermodynamics of the binding process, we have measured both activation and equilibrium free energies for the antibiotics and some derivatives. Equilibrium results are presented in Table 1, and were obtained by UV difference spectroscopy. In addition, we have measured $-\Delta G$ for ristocetin –Ac–D–Ala–D–Ala at pH 10 to be 4.4 kcal mol^{-1}. Although binding of N-acetylated derivatives of vancomycin and ristocetin can be detected by NMR in DMSO solution, the equilibrium constants for these compounds in aqueous solution were so low that no binding could be detected by UV difference spectroscopy. The same is also true for CDP-I. On the assumption that complexes of these compounds would produce similar UV difference spectra to those produced by the underivatised antibiotics, this means that the binding constants in aqueous solution must be no more than 10^3 $lmol^{-1}$.

Activation parameters were obtained by observation of coalescence of free and bound signals in the NMR spectrum, and are presented in Table 2. $\Delta G^{\ddagger}$ for the ristocetin –Ac–D–Ala–D–Ala complex at pH 10 was measured at 14.7 kcal mol^{-1} ($k_{off} = 113\ s^{-1}$).

Table 1. Thermodynamic parameters of peptide binding[a]

		Ac-D-Ala-D-Ala	Ac_2-L-Lys-D-Ala-D-Ala
Vancomycin	$-\Delta G$/kcal mol^{-1}	6.2 ± 0.1	8.5 ± 0.1
	$-\Delta H$/kcal mol^{-1}	7.6 ± 2	17.5 ± 2.5
	$-\Delta S$/e.u.	5 ± 7	30 ± 9
Ristocetin A	$-\Delta G$/kcal mol^{-1}	7.5 ± 0.1	7.8 ± 0.1
	$-\Delta H$/kcal mol^{-1}	7.5 ± 2	10.0 ± 2
	$-\Delta S$/e.u.	0 ± 7	7.3 ± 7

[a] Values estimated for 28°C. pH = 5.0.

Table 2. Activation parameters of peptide binding[a]

		Ac-D-Ala-D-Ala	Ac_2-L-Lys-D-Ala-D-Ala
Ristocetin A	$\Delta G^{\ddagger}$/kcal mol^{-1}	15.3 ± 0.3	16.3 ± 0.5
	$\Delta H^{\ddagger}$/kcal mol^{-1}	17.4 ± 2	18.7 ± 2.5
	$\Delta S^{\ddagger}$/e.u.	7.2 ± 5	7.8 ± 6
	k_{off}/s^{-1}[b]	41	7.7
	k_{on}/l $mol^{-1}s^{-1}$	1.2×10^7	3.8×10^6
Vancomycin	$\Delta G^{\ddagger}$/kcal mol^{-1}	11.0 ± 2	12.0 ± 2
	k_{off}/s^{-1}[b]	2×10^4	10^4
	k_{on}/l $mol^{-1}s^{-1}$	10^9	10^{10}

[a] Values estimated for 26°C.

[b] Errors in k_{off} about x 5 for ristocetin, x 50 for vancomycin. k_{on} calculated from k_{off} and ΔG (in Table 1).

DISCUSSION

The conformational change

This section deals with the question of why vancomycin and ristocetin undergo conformational changes on binding. At the end, the discussion is widened to consider the conformational changes undergone by proteins.

Thermodynamics. We have seen that the conformational change undergone by both antibiotics reduces their freedom of motion, and hence their entropy and the strength of the binding interaction. Vancomycin binds to Ac–D–Ala–D–Ala ("dipeptide") with a binding energy 1.3 kcal mol^{-1} less favourable than that of ristocetin to dipeptide (Table 1), and it is tempting to ascribe all of this to the greater entropy loss of the more flexible vancomycin leucyl sidechain. Certainly the difference in binding energy is essentially entirely due to entropy, but attempts to divide ΔG into ΔH and ΔS are notoriously beset by pitfalls.[1,20] We have shown above that ristocetin also loses considerable conformational freedom on binding, so the total loss of free energy due to the conformational change of vancomycin is probably greater than 1.3 kcal mol^{-1}.

The conformational change is unfavourable enthalpically as well as entropically. If we divide the binding process into the hypothetical steps antibiotic→antibiotic in bound conformation→complex, and consider the first step, we see that for ristocetin this means bringing up ring IV to shield the three adjacent NH groups from solvent, and thus reduce their ability to hydrogen bond to solvent. The first hypothetical step is thus unfavourable. For vancomycin, the conformational change is even less favourable, since it also serves to bring the three NH groups together, when enthalpically they would prefer to be as in the free conformation. If the antibiotics were already in the bound conformation naturally, these unfavourable processes would be avoided and the binding could be stronger.

Kinetics. There is however a very good reason why the antibiotics could not be much more rigid, which is that the on-rate† would be impossibly slow. Although the binding energy for ristocetin–dipeptide is 1.3 kcal mol^{-1} more favourable than that for vancomycin, its on-rate is about 100 times slower (Table 2). This is undoubtedly a consequence of the less flexible binding site of ristocetin, as a result of which the hydration shell around the carboxylate ion of the peptide must be partially removed before it can be inserted into the binding site (Fig. 5).

Our results show that the vancomycin on-rates are diffusion controlled. Diffusion-controlled reactions are faster for charged molecules than for neutral ones, and are proportional to the fraction of the surrounding space (the solid angle) to which the binding site is exposed[21] (Fig. 6). From the very fast rates observed here, we presume that the initial interaction is between the carboxylate and ammonium ions, with vancomycin in its normal "unfolded" conformation, thus allowing the ammonium ion to present the maximum solid angle to the approaching peptide. The remainder of the binding is then analogous to an intramolecular interaction, and happens very fast, with much reduced entropy demands. The free energy barrier to the on process is about 3 kcal mol^{-1}, which is typical of diffusion-controlled processes, and reflects the activation energy required to push the intervening solvent away.[21]

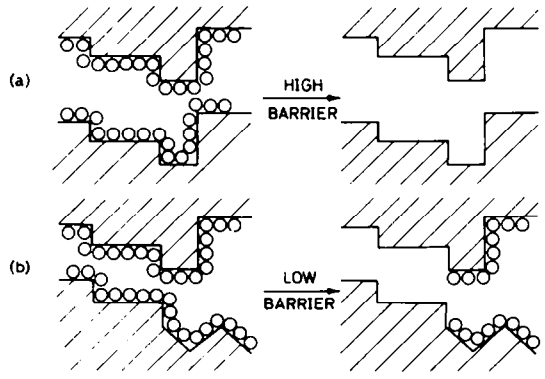

Fig. 5. Diagrammatic representation of the binding of a substrate (top) to a macromolecule (bottom); circles represent solvent molecules. In (a) the macromolecule is rigid, and the entire surface must be desolvated before binding can occur, giving a high activation energy barrier. In (b), the macromolecule is more flexible, and desolvation can occur in stages, with lower energy barriers and consequently a faster on-rate.

Proteins. The arguments given above for the kinetic necessity of conformational change apply equally well to proteins and other macromolecules. In fact it seems to be generally true that for antibodies[22] and other proteins[23] the on-rate is essentially the diffusion-controlled rate of roughly 10^8–10^{10} $lmol^{-1}$ s^{-1}, and the variety in binding constants comes from the variation in off-rates. This clearly makes sense for enzymes, giving them the maximum turnover rate, but also for binding proteins and antibodies, where the system is often not at equilibrium, and it is more important to remove substrate from solution quickly than to have the largest binding constant possible.

Clearly it remains true that the minimum loss of conformational freedom should occur on binding,

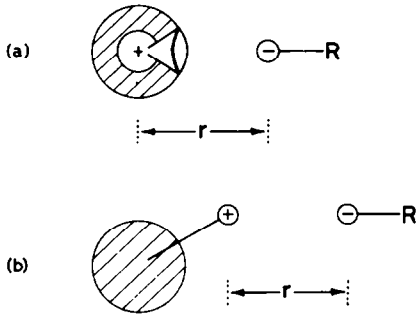

Fig. 6. Dependence of collision rate on solid angle. In (a) the macromolecular recognition site (positively charged) is buried in a cleft. The solid angle available for the negatively charged substrate to approach is indicated by the cone from the positive charge, and is small. The collision rate is therefore low. In (b) the positive charge is exposed, and the collision rate is faster.

†We define "on-rate" as the rate for the bimolecular reaction A + B→AB and the "off-rate" is the rate for the unimolecular reverse reaction. The binding constant is simply the ratio of the two; $K = k_{on}/k_{off}$.

consistent with diffusion-controlled on-rates. A good way of achieving this is to have a small flexible "hinge" region linking two essentially rigid domains. The loss of entropy on closure of the hinge is small, and easily compensated by the gain in entropy in releasing water which is hydrating polar groups or hydrophobic surfaces. One of many examples is the arabinose binding protein.[24]

The carboxylate binding pocket

The carboxylate binding pocket is the most striking feature of the binding site of both vancomycin and ristocetin. Comparison of the two antibiotics (Figs. 3 and 4) shows that the pocket is almost identical in both. It contains three NH groups, pointing directly at the bound carboxylate, which line the bottom of a hydrophobic region. Clearly the hydrophobicity is no accident—both antibiotics have the option to swing the hydrophobic sidechain out and expose the polar groups to water, yet they prefer not to. In fact, in vancomycin, swinging the sidechain out would permit a more linear salt bridge between carboxylate and amino group, yet it is clearly energetically preferable to have a weaker salt bridge in order to make a good hydrophobic pocket. We therefore examine first the reasons for the hydrophobic nature of the pocket.

The folding in of a hydrophobic sidechain-ring IV in ristocetin or the leucine sidechain in vancomycin—has three effects. Firstly, it removes a hydrophobic surface from contact with water. It is immaterial that the surface on which the hydrophobic sidechain lies is largely hydrophobic; the energy of the "hydrophobic bond" is derived not from attraction between hydrophobic groups but from the favourable entropy change caused by release of highly ordered water from hydrocarbon surfaces. Secondly, it removes water molecules hydrating the carboxylate ion and the NH groups to which it hydrogen bonds. And thirdly, it creates a region of low dielectric constant around the carboxylate and NH groups. Any quantitation of the effect in such an inhomogeneous environment would be beyond present techniques, but the hydrophobicity of the pocket will undoubtedly both strengthen electrostatic interactions, and raise the energy of charged species. These effects counteract each other, but it would appear that, in this case, the strengthening of electrostatic interactions dominates. The large binding energy derived from these interactions, and the relatively small destabilisation resulting from putting the charged carboxylate in a hydrophobic region, are a consequence of the ideal geometry of the binding site.

We now consider the "salt bridge" in more detail. In vancomycin, it is somewhat bent (Fig. 4) but this does not seem to affect its strength unduly, since deprotonation of the amino group leads to a sizeable reduction in binding energy of 1.4 kcal mol^{-1}.[25] This may be compared to a reduction of 1.5 kcal mol^{-1} found for deprotonation of an amine or protonation of a carboxylate in antibody-antigen interactions.[26] The effect of N-acetylation of vancomycin is much more dramatic; no binding can be detected by UV difference spectroscopy. This is not unexpected, since N-acetylation not only introduces steric hindrance, but also removes an NH group.

The "salt bridge" in ristocetin is much more interesting. Because of the stereochemistry here, there is no conventional salt bridge, since the amino group is about 5 Å away from the carboxylate and pointing away from it. However, it must play a considerable part in the binding interaction, since N-acetylation of ristocetin leads to a substantial reduction of binding. This large change in binding energy is harder to explain than that of vancomycin. Steric factors will undoubtedly be involved, though from models it would appear that they cannot have a very large effect. It is also difficult to see how a purely electrostatic interaction could contribute this much energy because the geometry is wrong (Fig. 3). We have measured the binding energy at pH 10, at which pH the amine is deprotonated, and found it to be −4.4 kcal mol^{-1} for ristocetin–dipeptide, so that 3.1 kcal mol^{-1} of binding energy are lost on deprotonation. The off-rate for this complex at pH 10 is 113 s^{-1}; the on-rate is therefore 1.8×10^5 $lmol^{-1}$ s^{-1}. Deprotonation has thus increased the off-rate by a factor of 3, which could be easily accounted for by electrostatic interactions; on the other hand, the on-rate has decreased by a factor of 100, and is the major contributor to the loss of binding energy observed. What does this tell us? We have seen above that the vancomycin on-rate is diffusion-controlled, but the ristocetin on-rate is slower because there is a higher activation energy barrier to be overcome to remove water hydrating the three NH groups in the hydrophobic cleft. These results imply that the initial interaction is between carboxylate and protonated amine, which would be expected to have a binding constant of about 0.4.[27] This weak complex then either dissociates or, more rarely, overcomes the activation energy barrier cited above and forms H-bonds to the NH groups in the hydrophobic pocket. Deprotonation of the amine weakens the initial interaction, producing a lower probability of overcoming the activation energy barrier. The reason why N-acetyl ristocetin has such a low binding energy is not clear, but similar factors may be involved.

In the transition state for the on process, the distance between carboxylate and amine must be increased to something like 5 Å, this being their approximate separation in the complex. It is perhaps significant that this distance is very close to the O–O next-nearest-neighbour separation of 4.9 Å found in pure water. It has been demonstrated that considerable stabilisation energy can be derived from H-bonding groups at this separation, sufficient for example to induce carbohydrates to adopt conformations in water different from those in other solvents.[28]

This work has a general relevance to protein-substrate binding. What we have shown is that hydrophobic binding sites are energetically favourable *per se*. Although, it has long been clear that preformed hydrophobic binding sites with a charged group at the bottom can bind strongly to oppositely charged species,[29] it has not been generally appreciated that it can still be favourable to take dipolar groups and charged groups, both from an aqueous environment, and put them together in a hydrophobic region. This conclusion is in agreement with calculations made by Warshel,[30] who has shown that ionic intermediates in enzyme-catalysed reactions can be stabilised up to 9 kcal mol^{-1} more by charged groups within the hydrophobic active site than they would be in water. The increased stabilisation de-

Table 3. Free energies for the combination of vancomycin and ristocetin with peptides[a]

Peptide	Vancomycin-ΔG	Ristocetin-ΔG
Ac_2-L-Lys-D-Ala-D-Ala	8.5	7.8
Ac_2-L-Lys-D-Ala-Gly	7.0	5.9
Ac_2-L-Lys-D-Ala-D-Leu	5.4	7.9
Ac_2-L-Lys-D-Ala-L-Ala	No combination	
Ac_2-L-Lys-Gly-D-Ala	6.8	7.1
Ac_2-L-Lys-D-Leu-D-Ala	7.4	6.5
Ac_2-L-Lys-L-Ala-D-Ala	No combination	
Ac-D-Ala-D-Ala	6.2	7.5

[a] Values at 26°C, in kcal mol^{-1}.

pends critically on correct positioning of the appropriate groups.

The initial charge–charge interaction between antibiotic and peptide makes for fast on-rates and facilitates the conformational change. It may be expected to be a fairly general feature of protein-substrate binding. The ristocetin salt bridge is so useful that it would be surprising if similar structures did not exist elsewhere. In particular, long-distance (5 Å) interactions between a solvent-exposed ionic group on the macromolecule and a substrate in a hydrophobic pocket seem likely to play a much more important part than is now appreciated.

Specificity

We have so far dealt with only a small part of the antibiotic, namely that part which binds the peptide carboxylate anion. The importance of the carboxylate binding site is obvious, yet a comparison of antibiotics in the vancomycin group shows that this binding site can be quite variable in detail, and other parts of the antibiotics are much more conserved; rings I, II, III, VI and VII are found in all the antibiotics, and are evidently vital to their success as antibiotics. These rings and their neighbouring atoms provide further non-covalent bonding interactions, but, more importantly, serve to confer *specificity* on the antibiotics. In this section we discuss the molecular origins of this specificity, and show once again how important correct hydrogen bond geometry is.

Table 3 presents some data for binding energies. The values are taken from Nieto and Perkins[16,17] except for the Ac-D-Ala-D-Ala and Ac_2-L-Lys-D-Ala-D-Ala results, which are taken from Table 1. We shall consider in turn how the binding is less effective for larger D side-chains; how L amino acids are excluded; and how the binding of a smaller sidechain (hydrogen) is reduced.

The reason why larger sidechains are bound less well is quite clear—they are too large for the holes available. This is probably the simplest and most effective of selection methods, and is also made use of by other molecules when extremely good selectivity is needed, such as for the t-RNA synthetases.[31] Some holes are a tighter fit than others, as exemplified by comparison of vancomycin and ristocetin. The effect of substituting D-Leu for D-Ala at the C-terminal amino acid (designated residue 1) is to reduce the binding energy by 3.1 kcal mol^{-1} for vancomycin, but there is no change in binding energy for ristocetin. Clearly the hole in vancomycin is made more selective for methyl groups by closing off one side with a chlorine atom. The same substitution at residue 2 now has more effect on ristocetin (1.4 rather than 1.1 kcal mol^{-1}) because one side of the hole in ristocetin is closed by an extra mannose residue[10] (Fig. 3).

Putting an L-amino acid in place of a D-Ala results in complete loss of binding. Clearly the pocket is completely the wrong shape for L-amino acids, as an inspection of models shows. Putting a D-amino acid in place of L-Lys reduces the binding energy by only 1–2 kcal mol^{-1}. The sidechain of L-Lys fits not so much into a pocket but rather along a hydrophobic surface,[10] and D-amino acids have enough freedom at position 3 that the sidechains can still make contact with the hydrophobic surface. In fact Ac-D-Ala-D-Ala-D-Ala binds more tightly to both antibiotics than does Ac-D-Ala-D-Ala. The conformational change of residue 3 which aligns the sidechain with the hydrophobic surface necessarily puts the amide bond leaving residue 3 into a different orientation relative to the antibiotic. This does not matter, because residues beyond 3 do not interact with the antibiotic. For residues 1 and 2, the orientation of the amide bond obviously does matter, and hence arises the intolerance for L-amino acids at these positions. On the basis of this discussion, we would expect it to be quite a general phenomenon that binding sites for peptides show considerable tolerance for the wrong chirality of the last amino acid on the binding site.

Substitution of glycine instead of alanine also results in a lower binding constant. It might be expected that a greater reduction in binding constant would occur in those binding sites into which methyl groups fit best, because more van der Waals and hydrophobic interactions would be lost, but this is not the case. The experimental data in Table 3 show that more binding energy is in fact lost when replacing Me by H in the *worst* (i.e. loosest) binding sites. We believe the most likely explanation for this is that putting a glycyl residue into a loose binding site allows a lot of motional freedom to the peptide. This motional freedom leads to a weakening of adjacent H-bonds, because the energy of a H-bond is

critically dependent upon its exact geometry. The greater weakening of H-bonds in less restricted binding sites predominates over the loss of dispersive or hydrophobic bonds in more restricted ones. It is therefore more important to maintain a good H-bonding geometry (a high "effective concentration" of H-bonding groups)[32] than to develop strong van der Waals or hydrophobic interactions. The strengths and geometries of hydrogen bonds are the most important determinant in the complicated balance of forces that decides whether binding will occur, and other bonding interactions will only occur if hydrogen bond strengths can be maintained. This is a conclusion also reached by other workers after consideration of protein structure.[33] We contend that the main reason why all antibiotics in the vancomycin group have an almost identical "left-hand half" is that this structure preserves the right distances between H-bonding sites to allow good binding. A most dramatic illustration of this is provided by the molecule CDP-I (2) in which there is an extra methylene group in the peptide backbone, slightly elongating the carboxylate pocket. Models would indicate that the difference this makes to the shape of the binding site is very small, and that it is possible to fit the peptide into the binding site, yet we have shown that CDP-I has little affinity for peptides.

CONCLUSIONS

The initial interactions between the antibiotics and peptide is between the peptide carboxylate and a protonated amine on the antibiotic. The amine should be as exposed as possible to the solvent, to allow a fast initial interaction. There is then a fast conformational change, in which the carboxylate falls into a binding pocket. In ristocetin, the conformational change leads to the amino group ending up 5 Å from the peptide carboxylate, but its presence is nonetheless important. An important feature of the carboxylate binding pocket is its hydrophobic walls. The non-aqueous environment strengthens dipole–dipole bonds, provided that the H-bond geometries are good. The rest of the antibiotic provides further bonding interactions to the rest of the peptide, and determines its specificity. Larger sidechains are excluded because they cannot fit into the binding site. We believe that the reason why glycyl residues are bound more weakly than alanyl is not only lack of bonding interactions involving the Me group, but also a weakening of the backbone H-bonds because of a looser fit into the binding site.

Many of these features are predicted to occur in protein-substrate interactions. The initial charge–charge interaction, the conformation change, the hydrophobic pocket, and above all the complementarity of H-bonding sites, are all so important here that they may be expected elsewhere. We note that conformational changes are necessary to provide acceptable on-rates, generally diffusion-controlled. They should therefore involve the minimum loss of flexibility. An important feature of a good binding site for a sidechain is that it fits the sidechain tightly. This means that the backbone amide groups will be held in the right place for good bonding, which is more important than van der Waals or hydrophobic bonding involving the sidechain.

EXPERIMENTAL

Materials. Vancomycin was purchased as "Vancocin HCl", a product of Eli Lilly, Indianapolis, and ristocetin A was a gift from Lundbeck, Copenhagen. Ac–D–Ala–D–Ala and Ac_2–L–Lys–D–Ala–Ala were synthesized as previously described.[10] CDP-I was prepared by the method of Johnson[34] (heating in water at pH 4.2 at 70° for 2 days) to give a microcrystalline sample which by NMR proved to be roughly a 2:1 mixture of the M and m isomers.[5] N-acetylvancomycin was prepared by acetylation of vancomycin by stirring in 4:1 MeOH:Ac_2O for 3 hr at room temp and evaporation to dryness. The product was purified by HPLC (Waters μC_{18} semi-prep column, 5% AcOH in ^{i}PrOH with linear gradient to 20%) to remove unchanged vancomycin and O-acetylated components. Di–N–Ac–vancomycin was characterised by ^{1}H NMR; the additional signals due to the two MeCO-groups appear at 1.92 and 2.10 ppm and the N–Me resonance is shifted downfield from its position in vancomycin to 2.90 ppm. It also gave the expected molecular ions in the positive ion mode in its fast atom bombardment (FAB) mass spectrum ($(M + H)^+$ at m/z 1532 and $(M + Na)^+$ at m/z 1554 in a thioglycerol/diglycerol matrix).

Ristocetin A was acetylated using a 1:1:2 mixture of Ac_2O, water and MeOH at room temp for 2 hr. By analytical reverse phase HPLC the product was largely (~90%) one component with a number of less polar impurities. The material had peaks at 1.89 and 1.93 ppm in the ^{1}H NMR spectrum which could be assigned to the two acetyl groups of di-N-acetyl–ristocetin A. The less polar components are presumed to arise from a small amount of O-acetylation and were not separated. The material gave a $(M + Na - 2H)^-$ ion at m/z 1271 in its FAB spectrum, using an aminoglycerol matrix.

Thermodynamic parameters. Equilibrium constants for the reaction antibiotic + peptide→antibiotic/peptide complex were determined by UV difference spectroscopy using a method based on that of Nieto and Perkins.[15] Experiments were carried out on a Pye-Unicam SP8-100 double-beam UV spectrophotometer with 4 cm pathlength cells and the bandwidth set at HE. The cells were thermostatted by water circulating through the cell holders from a constant temp bath. Solns of the antibiotics in 0.02 M sodium citrate, pH 5.0, were used at a concentration of 50 μM for titrations with dipeptide and 25 μM with tripeptide. The initial volume of soln in each cell was 10 ml. An aqueous soln of the peptide was added with a micropipette in small aliquots to the sample cell and an equal volume of water added to the reference. Concentrations of peptide used were 50 μM Ac–D–Ala–D–Ala and 10 μM Ac_2–L–Lys–D–Ala–D–Ala. The difference in absorbance, ΔA, developing on addition of peptide was monitored at a suitable wavelength; vancomycin/dipeptide 242 nm, ristocetin/dipeptide 246 nm, vancomycin/tripeptide 282 nm, ristocetin/tripeptide 287 nm. This difference was used as a measure of the amount of the complex formed assuming that the maximum value of the difference, ΔA_{max}, (reached on addition of a large excess of peptide) corresponds to 100% complex. The concentration of free antibiotic and of free peptide (C) at any point on the titration curve can be calculated assuming a 1:1 complex. The association constant was obtained from a Scatchard plot ($\Delta A/(\Delta A_{max} \cdot C)$ vs $\Delta A/\Delta A_{max}$) of the data. Points from above one equiv of peptide on the titration curve give a straight line for this plot, the slope of which is K. The intercept on the x-axis should give the stoichiometry of binding. The values obtained in our experiments varied between 1.0 and 1.2.

The free energy change due to binding was calculated using the equation $\Delta G = -RT \ln K$. The experiments were repeated at four temp between 28 and 65°. The enthalpy change for the reaction was estimated from a plot of log K *vs* 1/T and the entropy change obtained by difference using $\Delta G = \Delta H - T\Delta S$.

A value for the binding constant of ristocetin A at pH 10 was measured from a titration of a 50 μM solution of ristocetin A in 0.02 M sodium phosphate buffer at this pH with 50 μM Ac-D-Ala-D-Ala.

Activation parameters. Off-rates were measured by observation of coalescence temperatures,[35] T_c, for the C-terminal Ala Me signals at 100, 270 and 400 MHz, carried out on an XL-100 A, Bruker WH-250 and Bruker WH-400. Samples were about 6 mM in D_2O. The off-rate is given by $k_{off} = (\nu_F - \nu_B)\pi/\sqrt{2}$, where ν_F and ν_B are the frequencies of free and bound resonance at the coalescence temperature. These quantities were taken from spectra of free peptide and 100% bound peptide (a 2:1 ratio of antibiotic to peptide). The free energy is then given by $k_{off} = (kT_c/h)\exp(-\Delta G^{\neq}/RT_c)$, where k is Boltzmann's constant and the other symbols have their usual meaning. At different frequencies the Me signals coalesced at different temps, allowing $\Delta S^{\neq}$ and $\Delta H^{\neq}$ to be estimated. Temperature was measured at 100 MHz using a Digitron meter, at 250 and 400 MHz by observing the separation of the two signals of ethylene glycol, using the formula of Raiford *et al.*[36] For vancomycin, coalescence had not been reached even at 0°, but a broadening of the fast exchange peak was observed, and the coalescence temp was estimated by simple lineshape analysis to be a few degrees lower.

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