MOLECULAR PHARMACOLOGY, 11, 126-132

A Structure-Activity Study by Nuclear Magnetic Resonance of Peptide Interactions with Vancomycin

J. P. BROWN,[1] L. TERENIUS,[2] J. FEENEY, AND A. S. V. BURGEN

Division of Molecular Pharmacology, National Institute for Medical Research, Mill Hill, London, N.W. 7, England

Received July 30, 1974

SUMMARY

BROWN, J. P., TERENIUS, L., FEENEY, J. & BURGEN, A. S. V. (1975) A structure-activity study by nuclear magnetic resonance of peptide interactions with vancomycin. *Mol. Pharmacol.*, **11**, 126-132.

The interaction of vancomycin with peptides related to acetyl-D-alanyl-D-alanine has been examined by measuring the chemical shifts of all measurable protons on the peptide when they form complexes with vancomycin. The objective was to test whether alterations of the structures of neighboring groups are reflected in the interactions of an unchanged index group. In general this is so, the COOH-terminal alanine group showing least perturbation and the NH_2-terminal acetyl group showing the largest changes. The lability of the chemical shifts is a reasonable indicator of the contribution of the parts of the molecule to the over-all binding energy of the complex. When the structure of the ligand is changed, its topological relationship to the binding site changes so as to achieve a free-energy minimum. This makes linear addition of group free-energy components only an approximation.

INTRODUCTION

Structure-activity studies attempt to relate an alteration of binding affinity or some other, more indirect parameter of binding to a change in chemical structure. For instance, in a homologous series where there is replacement of a nonionizable proton by a methyl group, the changes in binding will be attributed to dispersion forces, hydrophobicity, or steric interactions. Usually the assumption is made in all but the last case that the additional forces introduced can be added to those existing in the parent compound. There may be cases in which this is at least partially true; however, in general it is probable that structural alterations of the ligand will lead to greater or smaller changes in the spatial relationship of the ligand and its binding sites as a result of optimization of all available interactions. Furthermore, if the ligand and binding site are not rigid, the binding might involve conformational changes which are also the consequence of the thermodynamic drive to the minimum energy state, and these also perturb any simple linear addition of free energy terms.

We have shown previously (1) that when the simple peptide *N*-acetyl-D-alanyl-D-alanine binds to the antibiotic vancomycin large chemical shifts occur in the proton resonances of the three methyl groups of the ligand. Nieto and Perkins (2), in an extensive study of the binding of small

[1] Recipient of a Medical Research Council scholarship, 1969-1972.

[2] Department of Medical Pharmacology, Uppsala, Sweden; supported by the Swedish Cancer Society.

peptides to vancomycin, established the structural criteria that determine the strength of the binding. It seemed to us that measurements of the chemical shifts which accompany the binding of these analogues might throw considerable light on the questions discussed above, since in many instances the chemical shifts reflect the environments of the protons and can be used to monitor small conformational changes.

EXPERIMENTAL PROCEDURE

The peptides were acetylated by the method described previously (1). Most peptides were purchased from Sigma Chemical Company or Cyclo Chemical Corporation. Three of the peptides (acetyl-D-Ala-D-Glu, diacetyl-L-Lys-D-Ala-D-Leu, and diacetyl-L-Lys-D-Ala-D-Tyr) were the generous gifts of Drs. H. R. Perkins and M. Nieto. The purity of all the peptides was checked by NMR measurements. Vancomycin hydrochloride was kindly supplied by Lilly Research Centre, Ltd., England. The NMR experiments were carried out using a Varian XL 100-15 NMR spectrometer operating at 100 MHz as described previously (1). For examining the α-protons in the alanyl peptides the *C*-methyl region was irradiated at a power sufficient to decouple the α-proton quartet to a single absorption band (see Fig. 1). All experiments were carried out in D_2O buffered to pD 5.5 (meter reading) in 0.05 M deuteroacetate buffer solution, and measurements were made at 34.5°.

Proton assignments were made from comparison of the observed chemical shifts with the shifts of model amino acids and peptides and, where necessary, from examining the effects of ionization on the chemical shifts. Chemical shifts were measured at different concentrations of vancomycin and the peptides; from these the binding constants and bound chemical shifts were obtained as described in the previous paper (1). The ^1H chemical shifts are reported in Hertz measured at 100 MHz.

In our discussion of the results, the residues are numbered from the COOH terminus; the α-protons have the suffixes "a" for the L-amino acid and "b" for the D-amino acid. These could not be assigned in glycyl peptides and are arbitrarily called "a" and "b" by analogy with the corresponding alanyl peptides.

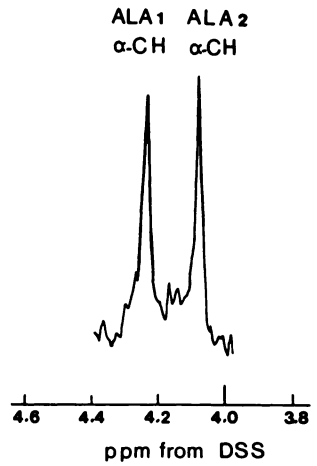

FIG. 1. *^1H spectrum at 100 MHz of α-CH protons decoupled from their adjacent CH_3 group in acetyl-D-Ala-D-Ala (3 mM).* [DSS is the reference, sodium 4,4-dimethyl-4-silapentane sulphonate.]

In all the complexes studied there was rapid exchange on the NMR time scale between the free peptide and peptide bound to vancomycin.

Origins of bound chemical shifts. The peptide bound chemical shifts result from interactions with groups in vancomycin (intermolecular) and from conformational changes within the molecule (intramolecular).

The intermolecular contribution to the bound chemical shift arises from the peptide groups coming into close proximity to anisotropic shielding groups, such as aromatic rings (ring current effects), or to charge groups (electric field effects). It has been established that there are five aromatic rings in vancomycin[3] which constitute over one quarter of the total structure. It is likely that in the binding site the peptide is in close proximity to one or more of these rings. Since even a single aromatic ring current system can produce large shifts (−2 to +4 ppm, depending on the distance and orientation from the proton)

[3] K. Smith and D. H. Williams, personal communication.

the potential of the five ring systems for producing shifts is considerable (4). Where shifts of greater than 1 ppm are produced, none of the other sources of shielding to be discussed is large enough to be held responsible. Electric field effects on the shielding could result from the charges of the NH_2-terminal *N*-methylleucine or the amino group of vancosamine. Such shielding would be expected when the peptide carboxylate group binds to the charged *N*-methylleucine fragment of vancomycin. If we assume that the shieldings of the peptide protons are influenced in a manner similar to that produced by protonation of the carboxylate anion, then the maximum effect predicted on the α-protons is a downfield shift of approximately 0.25 ppm (25 Hz at 100 MHz), while the α-CH_3 [as in Ala(1)] would be affected to less than 0.05 ppm (5 Hz at 100 MHz). Our analysis of pH effects on the complex of acetyl-D-Ala-D-Ala with vancomycin shows a trivial pH effect on the shielding of the two alanine methyl groups.

The intramolecular shielding effects arise from the fact that the proton shifts of the peptide are dependent on its conformation; the free peptide in solution exists as a mixture of rapidly interconverting conformers, and the observed chemical shifts correspond to the weighted average of the shifts of the various conformers present in solution. When the peptide forms a complex with vancomycin it is probably bound in a single conformation because of the constraints imposed by the binding. This conformational selection could lead to a chemical shift on binding. In glycine-containing cyclic peptides (3) the glycyl α-methylene protons have chemical shift differences of up to 0.2 ppm (20 Hz at 100 MHz); this provides some indication of the magnitude of the shielding effects from this source. The chemical shift difference between methyl protons of *N,N*-dimethylformamide enables one to estimate that the shift difference between α-protons that are either eclipsed by or *trans* to the carbonyl group in a glycine peptide will be about 0.3 ppm (30 Hz at 100 MHz). The effects on α-methyl protons will be much smaller than this, and probably less than 0.1 ppm.

These three contributions to the chemical shifts in the bound state are all capable of being influenced by the orientation of the peptide with respect to the binding site on the receptor. Where the relative positions of groups are conserved the shifts will be unaltered. On the other hand, a change in the bound shift provides unequivocal evidence that the spatial relationships have been disturbed.

RESULTS

The results obtained for six peptides for which it was possible to obtain reliable bound chemical shifts of all the nonexchangeable protons are summarized in Table 1 and are considered in detail below.

Acetyl-D-Ala-D-Ala. The pattern of shifts observed shows that the binding site provides a very complex magnetic environment for the peptide. CH_3(1a) is strongly shifted upfield by 57 Hz; yet H(1b), which is only distant by about 2.7 ± 0.9 A, has a shift of 4 Hz, so that the local field gradient is approximately 20 Hz/A. A similar situation exists with the second residue, CH_3(2a), which is shifted upfield by 20Hz, whereas H(2b) is shifted downfield by 23 Hz; the field gradient here is thus approximately 16 Hz/A. Both these residues should serve as very sensitive indicators for spatial relationships. The CH_3(3) group is shifted least of the three methyls.

Acetyl-L-Ala-D-Ala. No detectable binding was found between the peptide acetyl-D-Ala-L-Ala and vancomycin, in confirmation of the findings of Nieto and Perkins (2). However, acetyl-L-Ala-D-Ala does bind, although the binding energy is reduced by 2.3 kcal/mole compared with acetyl-D-Ala-D-Ala. The bound shift of CH_3(1a) is not significantly changed compared with that of the reference compound, but the bound shift of H(1b) is now −7 Hz, a downfield shift of 11 Hz. CH_3(2b) shows no shift, nor does CH_3(3), but H(2a) is shifted upfield by 20 Hz, a value similar to that found for CH_3(2a) in acetyl-D-Ala-D-Ala.

These results suggest that the CH_3(1a)

TABLE 1

Association constants and bound chemical shifts (at 100 MHz) of protons in peptides bound to vancomycin

Upfield shifts are positive. Errors are ±5%.

Peptide	K	Bound chemical shifts[a]								
		Residue 1a		Residue 1b		Residue 2a		Residue 2b		Residue 3:
		CH_3	H	CH_3	H	CH_3	H	CH_3	H	CH_3
	M^{-1}	*Hz*		*Hz*		*Hz*		*Hz*		*Hz*
Acetyl-D-Ala-D-Ala	14,000	+57			+4	+20			−23	+9
Acetyl-L-Ala-D-Ala	300	+60			−7		+20	0		0
Acetyl-Gly-D-Ala	6,000	+55			+7		+31		−46	+18
Acetyl-D-Ala-Gly	1,100		+132		−7	+20			−45	+5
Acetyl-Gly-Gly	130		+180		−33		+47		−80	+26
Acetyl-D-Ala	150	+56			−14	+5[b]				

[a] $\Delta = \Delta_{free} - \Delta_{bound}$.

[b] Acetylmethyl.

group has not altered its orientation with respect to the vancomycin binding site. The change in H(1b) shift value may reflect a different position of this proton; it could also be accounted for by a change in the conformation of the peptide required to allow Ala(1) to interact in the face of steric repulsion caused by the α-methyl group of Ala(2). The absence of a shift for CH_3(3) and CH_3(2b) on binding must be due to these groups being far away (>5A) from any of the aromatic groups in vancomycin. The +20-Hz shift of H(2a) could be due to its presence in a position similar to the average position of the three protons in the CH_3(2a) of acetyl-D-Ala-D-Ala, but it could also arise from a conformational change in the peptide. In acetyl-Gly-D-Ala, however, where a sterically forced conformation is unlikely, H(2a) shows a similar shift, suggesting that the first explanation may be correct.

It appears, then, that the insertion of the L-alanyl residue has little effect on the interaction of the terminal alanine residue 1 but that the acetyl group is no longer able to interact as a result of the inability of Ala(2) to fit the site.

Acetyl-Gly-D-Ala. In the free peptide the glycine methylene protons are accidentally equivalent and give rise to a single resonance; however, in the complex this becomes an *AB* quartet. In some of the ligand-vancomycin complexes the outer lines are not detectable and only the two strong inner lines of the *AB* system are seen. An *AB* system has been reported for some free glycine peptides and has been attributed to hindered rotation about the N—C_α bond and to the anisotropic shielding effects of the carbonyl group. The reported geminal coupling constants are close to 17 Hz (4–6). The shifts of the glycine resonances in bound acetyl-Gly-D-Ala have been analyzed assuming J = 17 Hz.

In bound acetyl-Gly-D-Ala the shifts for CH_3(1a) and H(1b) are not changed from those in the acetyl-D-Ala-D-Ala complex. However, the shifts of H(2b) and CH_3(3) are doubled, and that of H(2a) is larger than the value measured for acetyl-L-Ala-D-Ala.

These results could be explained most simply if the lack of the CH_3(2) group allowed the backbone of the acetyl glycyl-2 to move nearer the aromatic rings. It is clear that neither the acetyl group nor the backbone of residue 2 can be in the same position as in the bound reference compound.

Acetyl-D-Ala-Gly. In this peptide in the free state the glycyl methylene protons give a single resonance that becomes an *AB* quartet on binding. In residue 2 the shielding of CH_3(2a) is identical with that in the reference, but H(2b) is shifted downfield by another 22 Hz. CH_3(3), on the ot-

her hand, is decreased from +9 to +5 Hz. The H(1b) proton has a negative shift, and H(1a) shows a large upfield shift that can only come from the proton being in a stronger ring current field than the methyl protons of Ala(1) in the reference. The large shielding difference between H(1b) and H(1a) almost certainly implies that the glycyl moiety is bound in a rigid conformation. The chemical shift changes with respect to the reference compound in residues 2 and 3 show that the substitution of glycyl in position 1 affects the geometry of these residues with respect to the binding site.

Acetyl-Gly-Gly. In this case all the resonances are strongly shifted with respect to those in the reference. The glycyl methylene protons give *AB* quartets (Fig. 2), and the CH_3(3) has a bound shift of +26 Hz. H(1a) is shifted even further upfield than in bound acetyl-D-Ala-Gly, and H(1b) is shifted downfield by 37 Hz. Correspondingly large shifts are also found with H(2a) and H(2b). This experiment makes it clear

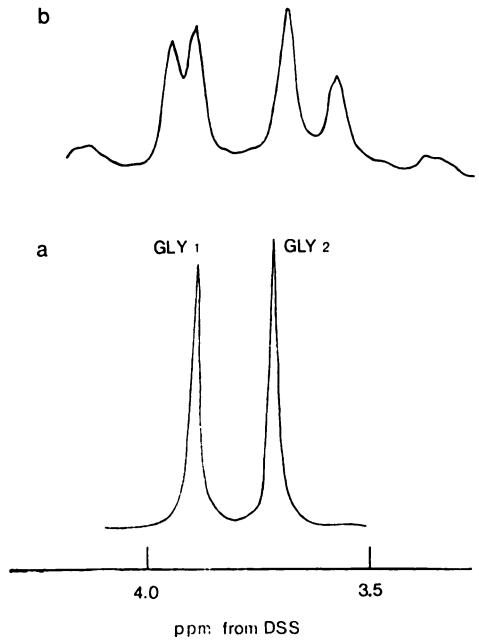

FIG. 2. *^1H spectrum at 100 MHz of acetyl-Gly-Gly in 10 mM solution at pH 6.0 (a) and 5 mM solution at pH 6.0 (b) in the presence of 1.25 mM vancomycin*

The glycyl methylene protons are now nonequivalent and give two *AB* quartets.

that the field in the binding site is strongly anisotropic, corresponding to 120 Hz/A for residue 1 and 72 Hz/A for residue 2. The radical change in shifts of H(1b) and H(2b) compared with the reference compound shows that the orientation of the peptide must be quite different from that in the acetyl-D-Ala-D-Ala complex. Energy minimization gives a binding energy of only −2.9 kcal/mole, compared with −5.7 kcal/mole for acetyl-D-Ala-D-Ala. The interaction energy of the two methyl groups must thus be in excess of −2.8 kcal/mole.

Acetyl-D-alanine. This acetylated amino acid binds as well as acetyl-Gly-Gly. The shift of the CH_3(1a) is the same as for acetyl-D-Ala-D-Ala, but H(1b) is shifted downfield by 18 Hz. Thus, while the CH_3(1a) binds at the same site as in the reference compound, the H(1b) position is influenced by a change in the peptide backbone allowed by the shortness of the ligand.

Table 2 gives data on seven peptides which have been studied in less detail.

D-Ala-D-Ala. Within the physiological pH range it was not possible to detect binding of this peptide. Since the positively charged NH_3^+ group of residue 2 might have prevented binding, we examined the reaction at pH 10, where the amino group is not protonated. Binding was demonstrated, and the binding constant (200 M^{-1}) was one-eighth of that for acetyl-D-Ala-D-Ala at this pH (1600 M^{-1}). Nevertheless the shifts of both CH_3(1) and CH_3(2) were somewhat lower than for acetyl-D-Ala-D-Ala, for which we have shown (1) that the bound shifts at pH 10 are no different from those at pH 5.5.

Acetyl-D-Ala-D-Glu and acetyl-D-Ala-D-Thr. Both these peptides are bound much more weakly than acetyl-D-Ala-D-Ala, presumably because the bulky side chains in residue 1 prevent a satisfactory fit with the sterically demanding subsite of the receptor. In neither case is the shift of CH_3(2) much changed, but CH_3(3) is larger in both instances and thus must reflect the displacement of the peptide backbone.

Acetyl-D-Ala-D-Ala-D-Ala and D-Ala-D-Ala-D-Ala. The addition of a further D-alanyl residue has only a small effect on the

TABLE 2

Association constants and bound chemical shifts (at 100 MHz) of protons in peptides bound to vancomycin
Upfield shifts are positive.

Peptide	K	Bound chemical shifts[a]			
		Residue 1	Residue 2	Residue 3	Others
	M^{-1}	*Hz*	*Hz*	*Hz*	*Hz*
Acetyl-D-Ala-D-Ala	14,000	57 ± 3 (αMe)	20 ± 1 (αMe)	8.6 ± 1 (AcMe)	
D-Ala-D-Ala (pH 10)	200	32 ± 3 (αMe)	9 ± 1 (αMe)		
Acetyl-D-Ala-D-Glu	600		26 ± 1 (αMe)	25 ± 1 (AcMe)	
Acetyl-D-Ala-D-Thr	380		18 ± 1 (αMe)	15 (AcMe)	
D-Ala-D-Ala-D-Ala	1,800	57 (αMe)	19 (αMe)	−3 (αMe)	
Acetyl-D-Ala-D-Ala-D-Ala	10,000	55 (αMe)	15 ± 5 (αMe)	10 ± 2 (αMe)	−7 ± 0.5 (AcMe)
Diacetyl-L-Lys-D-Ala-D-Leu	2,700		30 ± 4 (αMe)	−23 ± 0.5 (βCH_2) (γCH_2)	10 (αAcMe) −7 (ϵAcMe) −12 (δCH_2)
Diacetyl-L-Lys-D-Ala-D-Tyr	3,000	−43 (βCH_2)	35 ± 1 (αMe)		7 ± 0.5 (αAcMe) −6 ± 0.5 (ϵAcMe)

[a] $\Delta = \Delta_{free} - \Delta_{bound}$.

stability of the complex. The shifts of residues 1 and 2 are not significantly affected, showing that they bind in the same manner as in the reference complex. The acetylmethyl protons are shifted downfield, and the acetyl group is evidently still near an aromatic ring in vancomycin. Just as with the dipeptide, a free amino group depresses binding very markedly, although the shifts of CH_3(1) and CH_3(2) remain unchanged.

DISCUSSION

This system has proved to be useful for illustrating the problems involved in assuming, in structure-activity studies, that when one group in a ligand is changed, the topological relationship between the other groups and the receptor site is unaltered.

In this system it is clear that the position of binding of the methyl group of Ala(1) shows very little variation with structure of other residues, and presumably this group is located within a pocket in the receptor site in which little relative motion is permitted. The good fit of this part of the interaction is also indicated by the large effects on the energy of interaction produced by modifying this group. On the other hand, the position of the α(1)H probably varies more. The shift of the methyl of residue 2 is less constant and is affected by substitution, particularly in residue 1. This methyl group also appears to contribute less to the binding energy. It is likely that the position in the binding site for which it has affinity fits less well than that for residue 1. The shift of the *N*-acetylmethyl is very labile and can be affected by changes in residue 1 or 2. It is likely that the N-acetylmethyl residue may act as a sensitive indicator of quite small changes in the position and conformation of the peptide backbone.

Since intermolecular forces are highly sensitive to the distance between interacting atoms, the changes in the topology of the ligand when a group substitution is made must contribute to the net change of free energy of the complex. This cannot be attributed simply to the interactive energy of the index group. The results also suggest that a group which makes a large contribution to the interaction (e.g., the α-methyl of

residue 1) will be less subject to perturbation by substitutions than one whose contribution is small (e.g., the *N*-acetyl group). Provided that an indicator group can be shown to be in an anisotropic shielding field, the lability of its chemical shift in response to structural alterations can be used to assess its contribution to the stability of the complex.

CONCLUSIONS

A study of the chemical shifts of the nonexchangeable protons on acetyl-D-Ala-D-Ala when bound to vancomycin has shown that characteristic bound shifts can be found for each proton. The bound shifts are dominated by shielding contributions from intermolecular interactions with groups in the highly inhomogeneous binding site of the vancomycin, with smaller contributions resulting from intramolecular changes in peptide conformation. Modification of acetyl-D-Ala-D-Ala leads to changes in the shifts for the replaced amino acids, and frequently for adjacent groups (in some cases up to 2 residues away). By noting the bound chemical shifts which remain constant despite modifications at other residues in the peptide it is possible to define the parts of the molecule which bind in a similar position in the complex for different peptides.

It is concluded that the spatial relationship of the peptide to the vancomycin binding site depends on optimization of the binding of the whole peptide and connot be regarded as a linear addition of components.

ACKNOWLEDGMENT

We are grateful to Lilly Research Centre, Ltd., for a generous supply of vancomycin hydrochloride.

REFERENCES

1. Brown, J. P., Feeney, J. & Burgen, A. S. V. (1975) *Mol. Pharmacol.*, **11**, 000-000.
2. Nieto, M. & Perkins, H. R. (1971) *Biochem J.*, **123**, 789-803.
3. Kopple, K. D. & Ohnishi, M. (1969) *J. Am. Chem. Soc.*, **91**, 962-970.
4. Emsley, J. W., Feeney, J. & Sutcliffe, L. H. (1965) *High Resolution Nuclear Magnetic Resonance Spectroscopy*, Vol. I, Pergamon Press, Oxford.
5. Morlino, V. J. & Martin, R. B. (1967) *J. Am. Chem. Soc.*, **89**, 3107-3111.
6. Nagai, M., Nishioka, A. & Yoshimura, (1970) *J. Bull. Chem. Soc. Jap.*, **43**, 1323-1329.