Influence of kinetic barriers on the structures of non-covalently bonded states

Dudley H. Williams,* Ben Bardsley and Dominic P. O'Brien

Cambridge Centre for Molecular Recognition, University Chemical Laboratory, Lensfield Road, Cambridge, UK CB2 1EW. Fax: (+44) 1223 336913; E-mail: dhw1@cam.ac.uk

Received (in Cambridge, UK) 27th March 2000, Accepted 1st June 2000
Published on the Web 10th July 2000

The dissociation of two similar bimolecular complexes, of comparable thermodynamic stabilities, but different kinetic stabilities, is considered. Relative to the complex with the lower kinetic barrier to dissociation, it is concluded from proton NMR data that the complex with the higher kinetic barrier to dissociation has, at a defined point, a tighter interface and a less dynamic structure. The work illustrates how high kinetic barriers to dissociation of non-covalently bonded states can improve bonding at parts of the non-covalently bonded interface. The conclusions drawn should be of general relevance to the non-covalent complexes that are involved in biological functions.

Introduction

Vancomycin group antibiotics are the antibiotics of last resort in hospitals.[1] In Fig. 1, a general structure for dimers of the vancomycin group of antibiotics is given.[2] The dimers are important in increasing the activity of the antibiotics at the surfaces of bacteria.[3] We have recently demonstrated a correlation between the shortening of the distances between proximate atoms in the different halves of the peptide backbones of the dimers (Fig. 1) and their increasing thermodynamic stability.[4] The dimer interfaces become increasingly "tight" with increasing thermodynamic stability.

Specifically, we have described the relationship between the dimerization constant of the antibiotic dimers and the chemical shift of an antibiotic proton (known as x_4) which is located at the dimer interface (Fig. 1). The ^{1}H NMR resonance of this proton occurs at a more downfield shift when the antibiotic is dimerized than when it is a monomer (analogously to the downfield shift of an Hα-proton resonance in a β-sheet of a protein[5,6]). That the change in the chemical shift of x_4 is a good probe of the tightness of the dimer interface is supported by two further studies. First, it has been shown that in the formation of a β-hairpin, the downfield chemical shifts of Hα-protons (*cf.* x_4) are largely a result of inter-strand interactions.[7] This is evidenced first by the observation that a pronounced *i*, *i* + 2 periodicity is observed in the chemical shift changes such that inward-facing Hα-protons show the largest downfield shifts. Second, although an isolated strand of the hairpin is shown from coupling constant data and NOEs to preferentially occupy a β-strand, the downfield shifts of its Hα-protons are, relative to random coil values, very small. This latter finding also applies to other small peptides that form isolated β-strands.[8] Thus, in the present study, we can be confident that the downfield chemical shift of x_4 upon dimer formation is due to interactions with the other half of the dimer rather than to changes in backbone angles within any one antibiotic backbone. Such changes in backbone angles would in any event be severely limited since the antibiotics have relatively rigid backbones as a consequence of the tight coupling of the amino acid sidechains through covalent cross-linking.

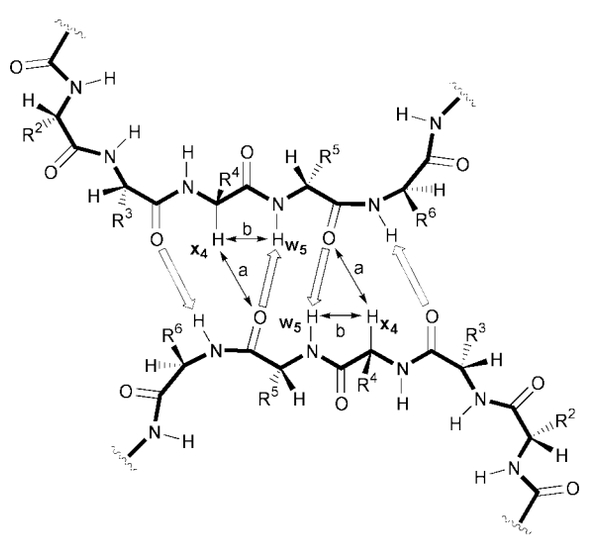

Fig. 1 Backbone structure of the vancomycin dimer. Hydrogen bonds at the dimer interface are indicated by thick arrows. The proton x_4, the chemical shift of which varies as a function of dimerization constant, and the adjacent amide proton w_5, are labelled. (a) Approach of x_4 to a carbonyl oxygen in the opposite half of the dimer leads to a downfield shift of its resonance upon dimerization. (b) NOESY cross peaks between x_4 and the resonance of the amide proton w_5 were observed and used in assignment of ^{1}H NMR x_4 monomer and dimer resonances.

The extent of the downfield shift of x_4 ($\Delta\delta_{x_4} = \delta_{x_4(\text{dimer})} - \delta_{x_4(\text{monomer})}$) increases as the distance of approach of the two halves of the dimer peptide backbone decreases, and has been shown to correlate with the magnitude of the antibiotic dimerization constant.[4] That is, the dimers become "tighter" at the peptide backbone interface (*i.e.*, they are formed with shorter hydrogen bonds) as they become thermodynamically more stable. For example, the dimerization constants of the antibiotics ristocetin-ψ, vancomycin, and chloroeremomycin are 50 M^{-1}, 700 M^{-1}, and 16,000 M^{-1}, respectively; the $\Delta\delta_{x_4}$ values for these antibiotics are 0.55 ppm, 0.70 ppm, and 0.78 ppm, respectively.[4]

This phenomenon of structural tightening of non-covalent complexes as a function of thermodynamic stability appears to be of some generality.[9–12] First, we note the increasing downfield shift of an NH antibiotic proton (bound to the CO_2^- group of bacterial cell wall analogues) as a function of increasing binding constant to the bacterial cell wall analogues.[9–11] This effect is found when the increasing downfield shift is

DOI: 10.1039/b002397n

induced either by extension of the hydrogen bond network[9,10] or through exercise of the hydrophobic effect.[11] Second, the downfield shifts suffered by carboxylic acid protons upon dimerization of aliphatic carboxylic acids increase with increasing dimerization constant of the acids.[12] The conclusion that structural tightening can be driven by the hydrophobic effect[11] is important since the hydrophobic effect is entropy driven at room temperature.[13,14] It therefore follows that entropic contributions that promote the favourable free energy of association can cooperatively promote the enhancement of neighbouring electrostatic interactions, *i.e.*, promote the exothermicity of formation of neighbouring hydrogen bonds.

In this study we report on an exception to the above trend (that, for a given system, structural tightness increases with increasing thermodynamic stability). Specifically, we show that the dimer of the vancomycin group antibiotic ristocetin A has a significantly tighter interface relative to the dimer of vancomycin, yet both dimers have comparable thermodynamic stabilities. We propose that the tighter interface in ristocetin A arises from the higher kinetic barrier to dissociation (relative to vancomycin). Our conclusions illustrate how kinetic barriers to dissociation can influence the structure of non-covalently bonded states. We also demonstrate how, for interactions with similar on-rates (association rates), the well established relationship between thermodynamic stability and structural tightening will hold.

Results and discussion

Dimerization kinetics of different members of the vancomycin group follow a trend which would be expected from inspection of their dimerization constants. Strongly dimerizing antibiotics, such as eremomycin and chloroeremomycin, exchange slowly from dimer to monomer on the NMR timescale (separate resonances due to monomer and dimer are therefore seen in their NMR spectra).[4] In contrast, the more weakly dimerizing antibiotics, such as vancomycin and ristocetin-Ψ, exchange rapidly from dimer to monomer on the NMR timescale and therefore only one set of time-averaged resonances is seen in the NMR spectrum.[4] These observations indicate that the dissociation rate (k_{off}) must be <50 s^{-1} for strongly dimerizing antibiotics and >400 s^{-1} for weakly dimerizing antibiotics at 300 K (based on measured chemical shift differences between monomer and dimer resonances and the observation of fast or slow exchange between those resonances). An exception to this trend is ristocetin A. The dimerization constant of ristocetin A is 500 M^{-1},[15] which is the same order as that of vancomycin (700 M^{-1}),[4] yet the two halves of its dimer exchange slowly on the NMR timescale.

The slow exchange (low value of k_{off}) of the ristocetin A dimer is correlated with a remarkably tight peptide interface in this dimer. This is indicated by the chemical shift of its x_4 proton. As described above, the magnitude of this parameter provides a qualitative measure of bond lengths across the dimer interface. A sample of ristocetin A (5 mM) was examined by proton NMR spectroscopy. At this concentration, approximately 65% of the antibiotic should be present as dimer and 35% as monomer, as calculated according to the dimerization constant of 500 M^{-1}. Resonances due to both monomer and dimer were therefore present in the ^1H NMR spectrum. Assignments of the resonances due to x_4 in the monomer and dimer forms of ristocetin A were made on the basis of cross peaks in the NOESY spectrum (τ_{mix} = 150 ms) to the amide proton resonance (known as w_5). This proton lies adjacent to x_4 in the structure of ristocetin A (Fig. 1). These assignments were consistent with the strong cross peak present between the monomer and dimer x_4 resonances which arises from chemical exchange between these two states during the mixing time (Fig. 2). The results of this experiment showed $\delta_{x_4(monomer)} = 5.46$ ppm and $\delta_{x_4(dimer)} = 6.36$ ppm giving a $\Delta\delta_{x_4}$ value of 0.90 ppm.

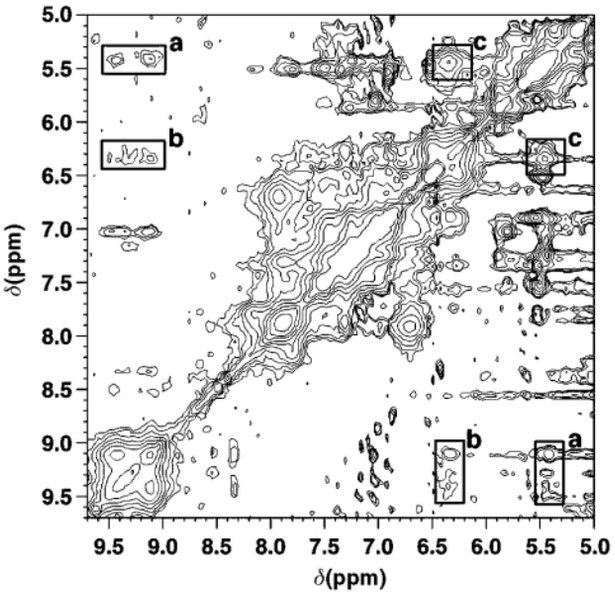

Fig. 2 Portion of the NOESY spectrum (τ_{mix} = 150 ms) of ristocetin A (5 mM in 9:1 H_2O–D_2O, pH 3.7, 300 K) showing cross peaks between the resonances of (a) x_4/w_5 in monomer and (b) x_4/w_5 in dimer, and (c) the exchange cross peak between the resonances of x_4 in the monomer and dimer states. As a result of chemical exchange, apparent cross peaks between x_4 and w_5 in different (*i.e.*, monomer and dimer) states are also observed.

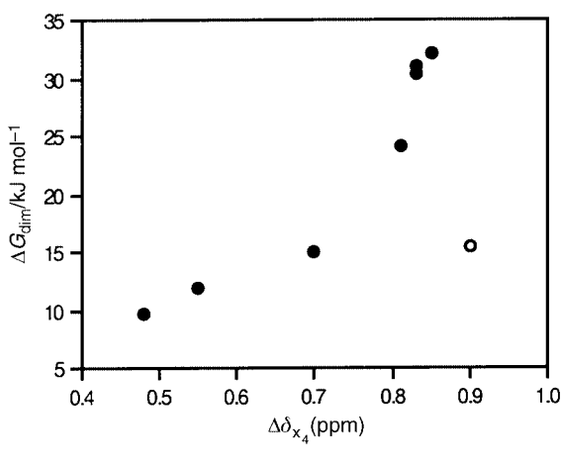

Fig. 3 Plot of the Gibbs free energy of dimerization (ΔG°_{dim}) *vs.* change in chemical shift of the proton x_4 upon dimerization ($\Delta\delta_{x_4}$) for some glycopeptide antibiotics. In order of increasing magnitude of ΔG°_{dim}, the filled circles represent dimerization of the following antibiotics: ristocetin-Ψ, monodechlorovancomycin, vancomycin, chloroeremomycin, eremomycin, biphenylchloroeremomycin and decaplanin. The open circle represents the dimerization of ristocetin A, which occurs with a much greater value of $\Delta\delta_{x_4}$ relative to ΔG°_{dim} than for other antibiotics.

This compares with a $\Delta\delta_{x_4}$ value for vancomycin of just 0.70 ppm under the same conditions.[4]

The remarkably large value of the $\Delta\delta_{x_4}$ value of 0.90 ppm for the ristocetin A dimer is emphasized by contrast with the $\Delta\delta_{x_4}$ value for ristocetin-Ψ (0.48 ppm).[4] Ristocetin-Ψ differs from ristocetin A only by removal of the tetrasaccharide attached to residue 4. The latter dimerizes only 10 times as strongly as the former (500 M^{-1} *vs.* 50 M^{-1}), yet the correlation between the free energy of dimerization of all the other antibiotics and $\Delta\delta_{x_4}$ values (Fig. 3) would suggest that a dimerization constant of *ca.* 6×10^8 M^{-1} would be necessary to produce such a tight interface of the two peptide backbones. That the remarkable change has occurred in the structure of the ristocetin A dimer (rather than in the structure of the monomer) is indicated by the chemical shift data. The monomers of ristocetin A and ristocetin-Ψ exhibit very similar x_4 proton chemical shifts (5.46

and 5.42[4] ppm, respectively), whereas the shifts for the dimers are very different (6.36 and 5.90 ppm, respectively).

Why are the peptide backbones of the two halves of the dimer of ristocetin A so intimately bonded given the relatively small dimerization constant of ristocetin A? The phenomenon appears likely to be related to the large kinetic barrier to dissociation of the dimer (in which respect the ristocetin A dimer behaves like the strongly dimerizing antibiotics chloroeremomycin and eremomycin). This large kinetic barrier (absent in ristocetin-Ψ) must be associated with the presence of the tetrasaccharide moiety of ristocetin A. This tetrasaccharide unit associates with a second tetrasaccharide moiety in the ristocetin A dimer.[16] Since there is a large interfacial area (peptidic *and* tetrasaccharide) in this dimer, there is a necessity for the two halves to move towards the transition state for dissociation without extensive solvation of the peptide backbones. This follows since the near-central parts of a large interface are necessarily remote from solvent, and the peptide backbone is indeed near the centre part of the interface. Hence, the kinetic barrier to dissociation is anticipated to be greater than in the case of vancomycin. This physical model is supported by the above experimental facts.

Yet it is clear that the ristocetin A dimer does not have the high thermodynamic stability found for chloroeremomycin and eremomycin. We conclude that: (i) the ring 4 disaccharide, the ring 6 amino-sugar, and the ring 2 chlorine atom, found in the latter two antibiotics, have a greater influence in promoting the thermodynamic stability of dimers[17] than does the tetrasaccharide of ristocetin A, but that: (ii) the large tetrasaccharide of ristocetin A is effective in promoting a large kinetic barrier to the dissociation of its dimer without causing a large increase in the thermodynamic stability of the dimer. This large kinetic barrier to the dissociation decreases the dynamics of the dimer peptide–peptide interface, with the result that this part of the dimer interface becomes more intimately bonded.

Our conclusions suggest that the thermodynamic stability of the complexes is not the sole factor governing complex "tightness" at the peptide–peptide interface. Instead, the kinetic barrier to dissociation of the complex ($\Delta G^{\ddagger}_{dim}$) must also play a role in determining the structure of the bound state. This of course raises the question as to why a correlation[4] between the dimerization constant (a thermodynamic parameter) and $\Delta\delta_{X_4}$ is otherwise observed and whether this correlation has any causal origin given that ristocetin A does not follow the trend of other antibiotics (Fig. 3). We propose (Fig. 4) that the "tightness" of the dimer structure is in the general case correlated with the depth of the Gibbs free energy well in which the dimer lies ($\Delta G^{\ddagger}_{dim}$). This proposal accommodates the otherwise anomalous behaviour of ristocetin A. However, the "tightness" of the dimer *will* correlate with ΔG°_{dim} in those cases where there are not large differences in k_{on} among the various dimerizing species (Fig. 4).

The way in which an increased value of $\Delta G^{\ddagger}_{dim}$ (relative to the vancomycin case) might be expected to restrain the dynamic motion at the peptide backbone of the ristocetin A dimer interface is illustrated in Fig. 5.

Conclusion

We have demonstrated how the intimacy of association of two non-covalently bonded entities (the "tightness" of the complex) is influenced by the ease with which the two entities can dissociate. The observation of differences in the structures of the ristocetin A and vancomycin dimers, despite their similar thermodynamic stabilities, illustrates the local interfacial tightening which can accompany a relatively high kinetic barrier to dissociation. This conclusion should have wide relevance to the non-covalent complexes which are responsible for biological function.

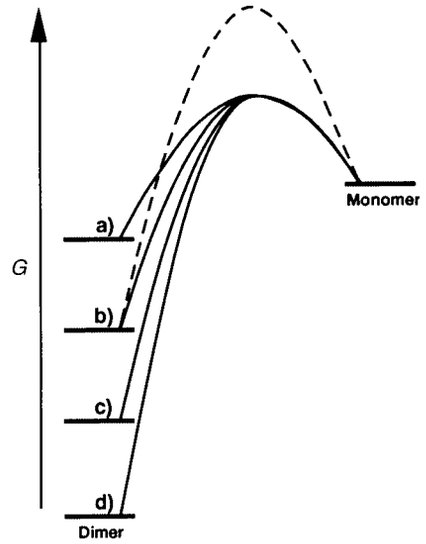

Fig. 4 Schematic Gibbs free energy pathways describing the monomer–dimer transition for a number of glycopeptide dimers with differing thermodynamic stabilities (ΔG°_{dim}). In order of increasing magnitude of ΔG°_{dim}, the pathways shown are for the antibiotics (a) ristocetin-Ψ, (b) ristocetin A (dashed line) and vancomycin (solid line), (c) chloroeremomycin, and (d) eremomycin. The solid lines illustrate, given similar on-rates, the increasing kinetic barriers ($\Delta G^{\ddagger}_{dim}$) to dissociation of the dimers which are associated with increasing thermodynamic stabilities. The dashed line for ristocetin A illustrates its unusually large barrier to the off-process (comparable in magnitude to those of the more strongly dimerizing antibiotics chloroeremomycin and eremomycin, as evidenced by slow exchange in the proton NMR spectrum).

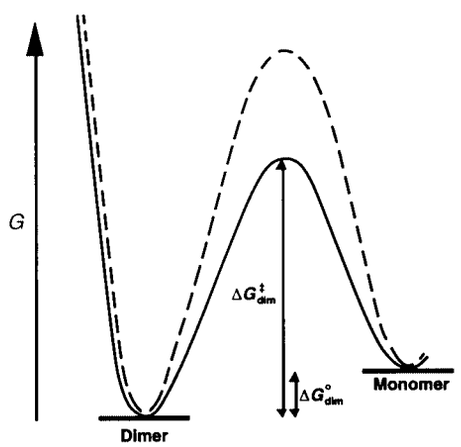

Fig. 5 Schematic Gibbs free energy pathways for the dissociation of the glycopeptide dimers ristocetin A (dashed line) and vancomycin (solid line). The thermodynamic stability of each dimer (ΔG°_{dim}) is approximately the same, yet ristocetin A dimerization occurs *via* a higher energy transition state (a higher free energy barrier to dissociation, $\Delta G^{\ddagger}_{dim}$). This leads to the proportionally greater occupation of bound states with free energies significantly below the transition free energy in the ristocetin A dimer (resulting in shorter interfacial bonds) than in the case of the vancomycin dimer (*i.e.*, ristocetin A has a narrower free energy well for dimer dissociation).

Experimental

Ristocetin A was obtained as a lyophilized powder from Abbott Laboratories (Chicago, USA) and used without further purification.

NMR spectroscopy

NMR samples were prepared as described previously.[4] Sample pD readings were measured with a Corning pH meter equipped with a combination glass electrode, and no corrections were

made for an isotope effect. NMR spectra were recorded on a Bruker DRX500 spectrometer at 300 K. Chemical shifts were measured with respect to internal sodium 3-trimethylsilyl-2,2,3,3-d_4-propionate. Water suppression was achieved using WATERGATE. Two-dimensional NOESY spectra were acquired in phase sensitive mode using time proportional phase incrementation (TPPI) to achieve quadrature detection in the indirect dimension. Spectra were recorded with 2048 complex data points in f_2 and 512 real data points in f_1 using a mixing time of 150 ms. Zero filling was used to give a final transformed matrix of 2048 × 2048 real points.

Acknowledgements

Abbott Laboratories (Chicago, USA) are thanked for a donation of ristocetin A. B. B. and D. O'B. thank the EPSRC for financial support.

References

1 S. Tabaqchali, *Lancet*, 1997, **350**, 1644.
2 J. P. Waltho and D. H. Williams, *J. Am. Chem. Soc.*, 1989, **111**, 2475.
3 D. H. Williams and B. Bardsley, *Angew. Chem.*, *Int. Ed.*, 1999, **38**, 1173.
4 D. H. Williams, A. J. Maguire, W. Tsuzuki and M. S. Westwell, *Science*, 1998, **280**, 711.
5 D. S. Wishart, B. D. Sykes and F. M. Richards, *J. Mol. Biol.*, 1991, **222**, 311.
6 G. Wagner, A. Pardi and K. Wüthrich, *J. Am. Chem. Soc.*, 1983, **105**, 5948.
7 A. J. Maynard, G. J. Sharman and M. S. Searle, *J. Am. Chem. Soc.*, 1998, **120**, 1996.
8 R. Zerella, P. A. Evans, J. C. Ionides, L. Packman, W. Trotter, J. P. Mackay and D. H. Williams, *Protein Sci.*, 1999, **8**, 1320.
9 P. Groves, M. S. Searle, M. S. Westwell and D. H. Williams, *J. Chem. Soc.*, *Chem. Commun.*, 1994, 1519.
10 M. S. Searle, G. J. Sharman, P. Groves, B. Benhamu, D. A. Beauregard, M. S. Westwell, R. J. Dancer, A. J. Maguire, A. C. Try and D. H. Williams, *J. Chem. Soc.*, *Perkin Trans. 1*, 1996, 2781.
11 G. J. Sharman, M. S. Searle, B. Benhamu, P. Groves and D. H. Williams, *Angew. Chem.*, *Int. Ed. Engl.*, 1995, **34**, 1483.
12 D. H. Williams, T. F. Gale and B. Bardsley, *J. Chem. Soc.*, *Perkin Trans. 2*, 1999, 1331.
13 C. Tanford, *The Hydrophobic Effect: Formation of Micelles and Biological Membranes*, Wiley, New York, 1980.
14 K. A. Dill, *Biochemistry*, 1990, **29**, 7133.
15 P. Groves, M. S. Searle, I. Chicarelli-Robinson and D. H. Williams, *J. Chem. Soc.*, *Perkin Trans. 1*, 1994, 659.
16 P. Groves, M. S. Searle, J. P. Waltho and D. H. Williams, *J. Am. Chem. Soc.*, 1995, **117**, 7958.
17 U. Gerhard, J. P. Mackay, R. A. Maplestone and D. H. Williams, *J. Am. Chem. Soc.*, 1993, **115**, 232.