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THE FREE ENERGY CHANGE OF RESTRICTING A BOND ROTATION IN THE BINDING OF PEPTIDE ANALOGUES TO VANCOMYCIN GROUP ANTIBIOTICS

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Abstract: The adverse cost in free energy of restricting an internal bond rotation in the binding of the peptide ligands N-succinyl-D-alanine and N-fumaryl-D-alanine to the antibiotic Ristocetin A, has been estimated to be 3.7±0.9 kJ mol^{-1} per rotor by comparison of the difference in free energies of binding measured by UV spectrometry.

The stability of a complex in aqueous solution, measured in terms of the free energy of binding, is the result of a balance of free energy components that work for or against a favourable association ($\Delta G<0$). In two recent publications[1,2] we have factorised the free energy of binding into a number of terms that have enabled us to analyse bimolecular associations between complementary peptide components. Our analysis is based upon the pioneering work of Jencks[3], and Page and Jencks[4], while a similar factorisation has been presented by Andrews et al.[5], and the relevance and physical basis of the terms involved have been summarised by Fersht[6]. In this paper, we concentrate on the orientational requirements for binding which necessitate the loss of degrees of freedom of internal rotation about bonds when functional groups within a flexible ligand become highly orientated in forming interactions with the complementary groups on the receptor. In this regard, we have considered an experimental approach that compares the free energy of binding of two peptide analogues (one with an additional restricted internal rotation) to ristocetin A, a member of the vancomycin group of antibiotics. We draw comparison with the free energy cost of restricting a rotation deduced by Page and Jencks[4] in a highly constrained covalent transformation, and with model systems based upon the melting of crystals of organic compounds.

N-succinyl-D-alanine (NSDA) and N-fumaryl-D-alanine (NFDA)[7] (Figure 1) represent analogues of the natural cell wall precursor that is the binding site of the vancomycin group of antibiotics. Introducing a double bound in NFDA enables us to assess the contribution of restricting a ligand rotor by comparison of the free energies of binding of NFDA with the saturated analogue NSDA. The interaction of these ligands with the binding pocket of ristocetin A is represented schematically in Figure 1, and is compared with that of a truncated form of the natural substrate N-acetyl-D-alanyl-D-alanine (NADADA).

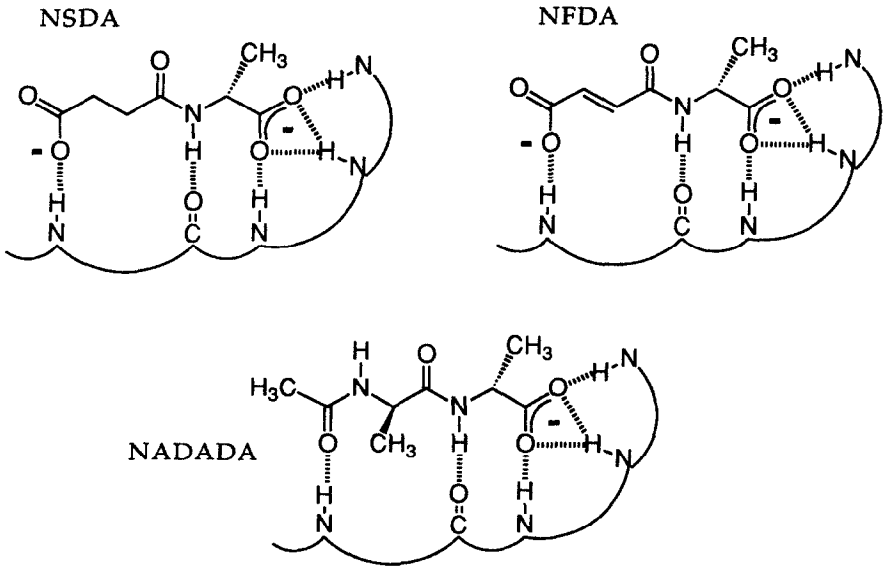


Figure 1: *Hydrogen bonds formed by NSDA, NFDA, and the cell wall analogue NADADA to the antibiotic ristocetin A*

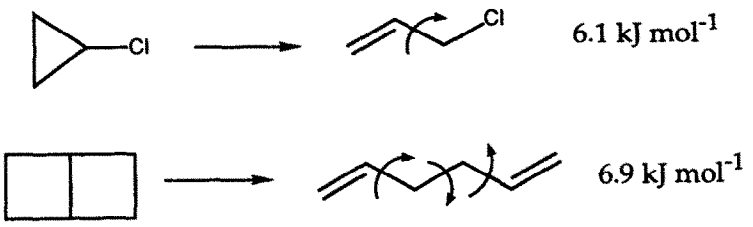
A notable difference is that NSDA and NFDA form a carboxylate/amide interaction at the ligand N-terminus instead of an amide/amide hydrogen bond as found for the natural substrate. Despite the structural differences, both NSDA and NFDA have been shown by detailed NMR studies[8] to bind in a completely analogous manner to that proposed for the N-acetyl-D-alanyl-D-alanine/ristocetin A complex[9].

In assessing the relative contributions that account for the difference in free energies of binding[10] of NSDA and NFDA of 5.5±1.3 kJ mol^{-1} [K_B (NSDA) 10000±1000 M^{-1}; and K_B (NFDA) 92000±10000 M^{-1}], we have estimated the relative change in the contribution from the hydrophobic effect to ligand binding by measuring the difference in solvent accessible non-polar surface area by rolling a water molecule of radius 1.4 Å over the non-polar surface. Despite the structural difference of a saturated versus unsaturated hydrocarbon chain, the effective solvent accessible surface area of NSDA and NFDA is calculated to be the same[11]. We conclude that the difference in binding energy between the two ligands does not reflect a difference in the contribution of the hydrophobic effect to binding. Additionally, both ligands bind with the same type and number of hydrogen bonds. Thus the difference of 5.5±1.3 kJ mol^{-1} in binding energy may be attributed to the different number of rotors restricted if the assumption can be made that both ligands show similar

van der Waals complementarity with the antibiotic binding site. The overall geometry of NSDA and NFDA are similar, but bond lengths and angles are affected by introducing a double bond into the ligand but the energy minimised structures of the ligand/antibiotic complexes (using MacroModel[11]) show that the average hydrogen bond lengths in both complexes are comparable (within ± 0.2 Å).

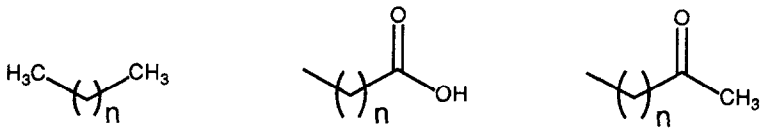
The difference in pK_a of succinic and fumaric acid (succinic acid, pK_{a1} = 4.0, pK_{a2} = 5.2; fumaric acid, pK_{a1} =2.8, pK_{a2} = 4.0, 25°C, ionic strength 0.1-1 M [12]) is a potential source of difference in the strength of the hydrogen bond involving the N-terminal carboxylate group. The charge density on the carboxylate group of NFDA is likely to be diminished by delocalisation within the conjugated π-system. These two effects are probably small but they oppose the binding of NFDA compared to NSDA. Therefore the experimental result of 5.5±1.3 kJ mol^{-1} can be regarded as a probable *lower* limit for the cost of restricting the additional rotation.

The introduction of the double bond in the fumaryl residue is also likely to increase the barrier to rotation of other conjugated bonds such that the overall number of rotors is potentially larger than one. Bonds may be described in terms of their percentage of double bond character from an analysis of bond lengths[13-15]. For example, the π-bond order of the single bond in butadiene has been calculated by various authors to be approximately 0.26±0.06, whereas that of the double bonds is ca. 0.96±0.02[13]. Similar results were reported for acrolein[14]. We conclude that each of the single bonds flanking the unsaturated linkage in NFDA probably also have a double bond character of about 0.25. Thus, in considering the binding of NFDA versus NSDA to ristocetin, the number of additional bonds restricted in binding the latter approximates to 1.5. On the basis of this analysis, the experimental difference in binding energy of 5.5±1.3 kJ mol^{-1} can be considered as resulting from an adverse free energy per rotor restricted of ca. 3.7±0.9 kJ mol^{-1}. Even the upper limit within the estimated range for the cost of restricting a rotation is less than the 5-6 kJ mol^{-1} per rotor derived by Page and Jencks[4] for "freezing" a rotor. This seems to reflect the fact that molecules held by weak non-covalent interactions probably retain considerably more (entropically favourable) residual motion on binding than molecules involved in highly constrained covalent transformations. The following examples of isomerisation reactions illustrate the potential limiting values for rotor restriction:



The value of TΔS per rotor restricted at 300K is obtained by dividing the total entropy change for the isomerisation by the change in the number of rotors; to a first approximation, we associate the net entropy change with the increase in the number of "free" rotors in the product, which makes the forward reactions entropically favourable. In the examples presented[16], bond rotations within the cyclic starting structures are highly restricted, resulting in large entropy changes in forming the unrestrained product of 6-7 kJ mol^{-1} per rotor, consistent with the conclusions of Page and Jencks[4], but considerably larger than found from the comparison of the binding of NSDA and NFDA to ristocetin A.

An alternative model for ligand dissociation from a complex is that presented by the melting of a crystalline substance in which degrees of internal flexibility are considerably restricted in the crystal lattice compared with internal motions in the liquid melt. In this regard we have considered the relationship between the entropy of fusion of hydrocarbon crystals and the number of rotors for homologous series of linear alkanes, alkyl carboxylic acids and 2-methyl ketones.[17]



Good linear correlations are found within these series (correlation coefficient >0.95) that give the entropic cost of restricting a rotor (TΔS at 300K) as between 2.3 and 3.6 kJ mol^{-1}. [17,18] The crystals are stabilised by non-covalent interactions and yield an entropic cost per rotor (= free energy cost per rotor) of 3.0±0.7 kJ mol^{-1} that bears reasonable comparison with the value of 3.7±0.9 kJ mol^{-1} deduced from the binding of the peptide ligands to ristocetin. Since organic crystals are held together by relatively weak intermolecular (rather than covalent) forces, their formation from melts can provide useful guides to entropy changes in the formation of weakly bound molecular recognition complexes that appear to be consistent with data from studies of molecular associations in aqueous solution.

Estimating the cost of restricting a rotation in a flexible molecule when it binds to a targeted receptor is an important parameter in our goal to develop an approach that will enable us to predict approximate free energy changes in molecular recognition events based on a partitioning of free energy contributions.

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7. N-succinyl-D-alanine (NSDA) and N-fumaryl-D-alanine (NFDA) were both prepared from D-alanine methyl ester, and mono-methyl succinate or mono-methyl fumarate, respectively. The N-succinyl-D-alanine dimethyl ester (N-fumaryl-D-alanine dimethyl ester) was prepared by stirring a 1:1 mixture of D-lanine methyl ester and mono-methyl succinate (mono-methyl fumarate) with N-methylmorpholine, hydroxybenzotriazole and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (8:1:2) in dichloromethane for 10 hours at room temperature. The solvent was evaporated and the residue dissolved in ethyl acetate. The solution was washed with water, sodium hydrogen carbonate solution, citric acid solution, and again with water, dried over potassium carbonate, filtered, and the solvent then evaporated under vacuum. Final purification by flash column chromatography using silica gel/ethyl acetate gave pure N-succinyl-D-alanine dimethyl ester (N-fumaryl-D-alanine dimethyl ester) (typical yields were 50-70%).
   N-succinyl-D-alanine dimethyl ester (N-fumaryl-D-alanine dimethyl ester) was dissolved in tetrahydrofuran and stirrred at 0°C while an excess of 2M lithium hydroxide solution was added. The mixture was stirred at room temperature for 5 hours. The solvent was evaporated and the residue dissolved in water. The solution was loaded on to a Dowex 50 (protonated form) ion exchange column and eluted with water until the pH of the eluate was neutral. Evaporation of water gave N-succinyl-D-alanine (N-fumaryl-D-alanine) (typical yields are 70-80%). Both ligands isolated in this way were shown to be pure by tlc (butanol/water/acetic acid 3:1:1; N-succinyl-D-alanine R_f 0.41; N-fumaryl-D-alanine R_f 0.54) and ^1H NMR spectroscopy. These products were hydrolysed (6N hydrochloric acid, 105 °C, 12h) to D-alanine, and shown to be optically pure (>96%) by chiral glc (Chirasil-Val) as N-trifluoroacetyl isopropyl ester derivatives.
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10. UV spectra were recorded on a UVIKON 940 dual beam spectrometer at 300±3 K. Antibiotic and ligand solutions were buffered with KH_2PO_4 (0.05 M)/NaOH (0.029

M), pH 7.0. The initial concentration of ristocetin A was 0.1mM. The λ_{max} for the complexes were determined from a 4-cell tandem arrangement in which the two cells in the reference beam contained the ligand and the antibiotic solutions respectively, while the two cells in the sample beam contained a mixture of the ligand and the antibiotic solutions, and buffer solution, respectively. Antibiotic concentrations in different cells were the same, as were ligand concentrations. The λ_{max} for both ligands was determined to be 286nm. Titration was carried out at the previously determined wavelength in a 2-cell arrangement, where buffer and ristocetin A solutions were placed in the reference and sample beam respectively. Equal aliquots of the ligand solutions were added successively to both cells, followed by stirring (2 minutes) and measurment of the absorbances.
Binding constants were derived from experimental results using scatchard plots and Simplex least squares curve-fitting program (Press, W.H.; Flannery, B.P.; Tenkolsky, S.A.; Vetterling, W.T.; *Numerical Recipes in Pascal*, (1989), Cambridge University Press.

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18. We exclude from the analysis the series of alkanes with an odd number of carbons on the basis that crystals of these hydrocarbons undergo solid-solid phase transitions, which are not observed for the even-series alkanes. Thus, just below the melting temperature the odd-series hydrocarbons are less well-ordered and this is reflected in a smaller favourable entropy change ($T\Delta S$ at 300K) per rotor on melting of 1.6 kJ mol^{-1}.