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The Energetics of Intramolecular Reactions and Enzyme Catalysis

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Phil. Trans. R. Soc. Lond. B 1991 **332**, 149-156
doi: 10.1098/rstb.1991.0043

The energetics of intramolecular reactions and enzyme catalysis

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SUMMARY

The relative rates of reactions should always be examined by an awareness of differential effects. The magnitude and variation of the relative rates of intramolecular reactions can be rationalized by the differences in entropy and strain energy. The relative rates of enzyme-catalysed reactions are sometimes due to groundstate effects. The β-lactamase-catalysed hydrolysis of β-lactam antibiotics my require a unique disposition of catalytic groups owing to an unusual process of bond fission in the four membered ring.

1. INTRODUCTION

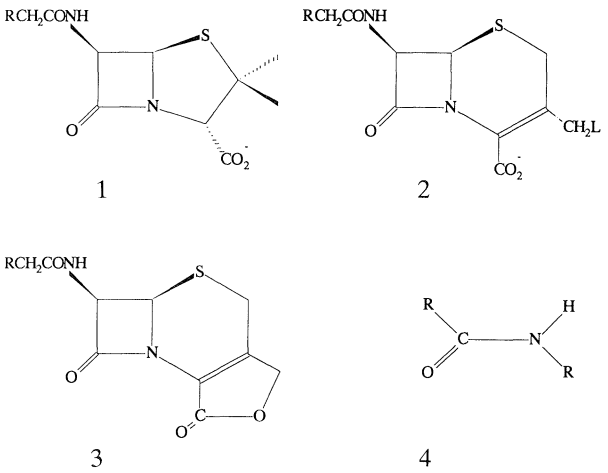
The beauty of chemistry and biology is that both are largely concerned with the study of change. Even the most sophisticated attempts to investigate an isolated and apparently static state usually involve a perturbation of that state and cause a change in the system. This is the dilemma of the philosophers, so what hope is there for understanding the process of the changes in bonding between atoms when a chemical reaction occurs? Apart from perhaps isotopic substitution, most studies of reactions involve a comparison between different systems and a significant perturbation of the system under investigation. We change the solvent, the temperature, the salt concentration, the substituents in both the substrate and the enzyme and then study the change these perturbations cause on some parameter involving a measurement of the change of state, such as a rate or equilibrium constant. It is not surprising therefore that in all these changes we often lose sight of the fundamental differences between the various states. Relative rate and equilibrium constants can be the result of changes in the energies of the initial state and the transition state or product state, respectively.

2. THE DIFFERENCE BETWEEN INTRA- AND INTERMOLECULAR REACTIONS

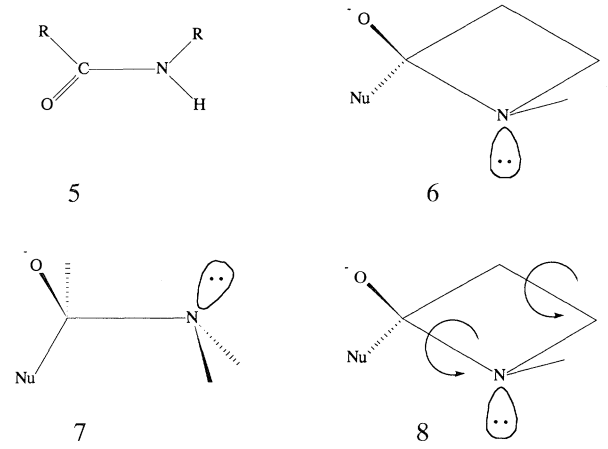
The first step in an enzyme-catalysed reaction is the bringing together of the substrate and enzyme. This binding process brings, in most cases, the reacting groups on the enzyme and substrate into close proximity (scheme 1). This approximation of reactants changes their molar free energy and has long been thought to be an important contribution to the efficiency of enzyme catalysis. An analogy is thought to be the efficiency of intramolecular reactions, in which the reactants are covalently linked together, compared with an intermolecular reaction between similar reactant molecules (scheme 1) (Page 1973; Capon & McManus 1976; Kirby 1980; Illuminati & Mandolini 1981; Mandolini 1986).

Even when nucleophilicities, electrophilicities, acidities and basicities etc. are the same in both the intra- and the intermolecular reaction, the former invariably occurs with a greater rate or equilibrium constant. Typical rate enhancements and favourable equilibria of intramolecular reactions are shown in scheme 2 (Capon & McManus 1976). All of these reactions involve the formation of five membered rings either in the product or transition state. However, a comparison of the equilibrium or rate constants for these reactions compared with analogous intermolecular reactions gives ratios varying from 0.5 to 2×10^{12} mol dm^{-3}. The rate enhancements and favourable equilibria have units of concentration because a unimolecular reaction is being compared with a bimolecular one. For this reason the rate enhancement is sometimes called the 'effective concentration' or 'effective molarity', which is the hypothetical concentration of one of the reactants in the intermolecular reaction required to make the intermolecular reaction proceed at the same rate or to the same extent as the intramolecular one. Immediate questions about these effective molarities are why the difference between intra- and intermolecular reactions and why the difference between the relative efficiencies of intramolecular reactions? There has never been a shortage of answers put forward to explain these phenomena and to extrapolate them to theories of enzymic catalysis (see table 1).

Some of these special explanations of the efficiency of intramolecular reactions assume that it is a *rate* phenomenon. However, the variation in the effectiveness of intramolecular reactions is shown by both equilibrium and rate constants (scheme 2), and furthermore, there is often a linear relation between



Scheme 1



Scheme 2

these constants (Page 1977). Attempts to explain the fast rates of intramolecular reactions by critical distances, critical angles, reaction windows, enhanced methods of energy transfer and other phenomena concerned with the rates of reactions are therefore not addressing the full problem.

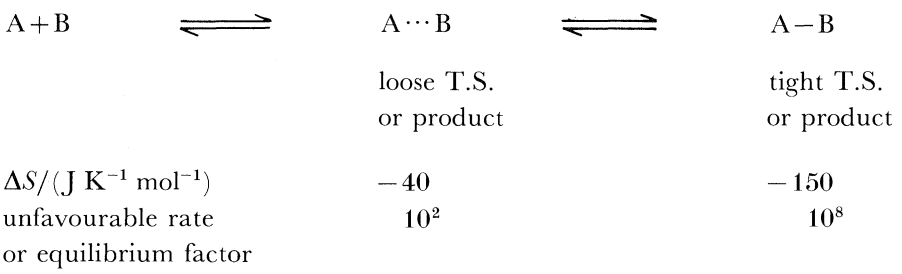
Changes in equilibrium constants may be understood by examining the variations in the free-energy difference between reactants and products. This thermodynamic approach is enhanced by a knowledge of the nature of reactants and products so that enthalpy and entropy differences can be related to structure. A similar approach may be used for the rates of reactions if the transition state theory of reaction rates is accepted. Although the structure of the transition state is not known directly, transition state theory allows the application of thermodynamics to understanding the relative rates of reactions.

Table 1. *Examples of explanations given for the efficiency of intramolecular reactions*

explanation	reference
entropy	Page & Jencks (1971)
approximation, propinquity, proximity	Jencks (1969); Bruice & Benkovic (1965); Koshland (1962)
togetherness	Jencks & Page (1972, 1974)
rotamer distribution	Bruice (1970)
anchimeric assistance	Winstein *et al.* (1953)
distance distribution function	De Lisi & Crothers (1973)
orbital steering	Storm & Koshland (1970, 1972); Dafforn & Koshland (1971)
stereopopulation control	Milstein & Cohen (1970)
substrate anchoring	Reuben (1971)
vibrational activation	Firestone & Christensen (1973)
vibrational activation entropy	Cook & Mckenna (1974)
orbital perturbation theory	Ferreira & Gomes (1980)
group transfer hydration	Low & Somero (1975)
electrostatic stabilization	Warshel (1978)
electric field effect	Hol *et al.* (1979); Van Duijnen *et al.* (1979)
catalytic configurations	Henderson & Wang (1972)
directed proton transfer	Wang (1970)
coupling between conformational fluctuations	Olavarria (1982)
gas phase analogy	Dewar & Storch (1985)
torsional strain	Mock (1976)
circe effect	Jencks (1975)
spatiotemporal postulate	Menger & Venkataram (1985); Menger (1985)
FARGE (freezing at the reactive centres of enzymes)	Nowak & Mildran (1972)

Large effective molarities are usually the result of a ground or reactant state effect simply because the molar free energy of the intramolecular reactant is greater than that of the intermolecular reactants owing to an entropy difference.

Bringing two molecules together is accompanied by a negative change in entropy because of the reduced volume of space available to the reactants. Mechanically, the increase in order in this process is expressed



Scheme 3

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mainly as a loss of translational and rotational entropy. The more severely the reactant molecules are confined when brought together the greater is the loss of entropy (Page & Jencks 1971; Page 1973). For most molecules of average shape and size this entropy change makes both the rates and equilibria of bimolecular reactions unfavourable by factors of up to 10^8 M at a standard state of 1 M and at 25 °C (scheme 3).

These changes do not occur in unimolecular reactions and 10^8 M is therefore the maximum difference between an intra- and intermolecular reaction based only on the entropy difference between them and in the absence of strain and solvation effects (Page & Jencks 1971; Page 1973). These calculated entropy changes will not necessarily be reflected in the observed entropies of activation and reaction because the experimental values are dominated by solvent effects especially in water and other polar solvents (Page & Jencks 1971; Page 1973, 1977).

Effective concentrations greater than 10^8 M are the result of strain-energy differences between the two systems being compared. Either the intermolecular reaction shows an unfavourable change in strain energy or the intramolecular one exhibits a release of strain upon ring closure (Page 1973, 1977). It has been shown that there is a good relation between rates and equilibrium constants for intramolecular reactions and the strain-energy changes accompanying ring closure (Page 1977). There is therefore nothing special about these very high effective concentrations and their relevance to enzymic catalysis is limited. Enzymes do not generally owe their efficiency to their ability to induce geometrical strain into their substrates (Page 1984).

The reasons why effective molarities vary, even for reactions involving formation of the same ring size, and are sometimes small, are as follows (Page & Jencks 1971; Page 1973, 1977): (i) unfavourable potential energy changes accompanying the intramolecular reaction, i.e. strain energy is introduced upon ring closure; (ii) unfavourable negative entropy changes in the intramolecular reaction resulting from the loss of internal rotation and a small loss of overall rotational entropy upon ring closure; the loss of internal rotation corresponds to a factor of only about 5–10 per internal rotation, and (iii) favourable positive entropy changes in the intermolecular reaction, resulting from a loose transition state, i.e. a weakly defined geometrical relation between the reactants. The entropy associated with the low frequency vibrations of such flexible transition states compensates for the large negative loss of entropy associated with translation and rotation to give a smaller unfavourable entropy change for bimolecular reactions (scheme 3).

In a series of recent articles it has been suggested that the rates of intramolecular reactions are very dependent upon the distance between the reacting atoms and the time spent at the 'critical distance' at which reaction occurs (Menger 1985; Menger & Glass 1980; Menger & Venkataram 1985). It is claimed this 'spatiotemporal' hypothesis shows that conventional entropy and strain arguments cannot explain the observed magnitude and variation in effective molarities of intramolecular reactions. However, these descriptions of reactions take no account of the fact that the efficiency of intramolecular reactions is not solely a rate phenomenon – it is reflected also in favourable equilibrium constants. Furthermore, 'the time that reactant molecules reside within a critical distance' is reflected by the entropy of the system. The 'spatiotemporal hypothesis' is a euphemism for entropy. The relative rates of the intramolecular reactions used to justify the spatiotemporal hypothesis may be explained by differences in strain energy and entropy (Page & Jencks 1987).

3. THE DIFFERENCE BETWEEN ENZYME- AND NON-ENZYME-CATALYSED REACTIONS

Because enzymes increase the rate of reactions and show discrimination between possible substrates there has been a temptation to treat these two phenomena separately. Classically the rate enhancement is often attributed to the chemical mechanism used by the enzyme to bring about transformation of the substrate. The fidelity of enzyme and substrate is accounted for by binding – as in the analogy of the 'lock and key'. It is now apparent that these simple ideas need to be reappraised. Chemical catalysis alone cannot explain the efficiency of enzymes. The forces of interaction between the non-reacting parts of the substrate and enzyme may also contribute to a lowering of the activation energy of the reaction (Page 1990). Catalytic groups of equal nucleophilicity and electrophilicity, etc. to those used by the enzyme cannot increase the rate of reactions to even a minute fraction of that achieved by enzymes in the absence of the rest of the protein structure.

The binding energy between substrate and enzyme may be used in a variety of molecular mechanisms to lower the activation energy of the reaction such as charge neutralization, desolvation, geometrical and entropy effects.

It is now generally considered that maximum binding energy, i.e. stabilization, occurs between the substrate and enzyme in the transition state of the reaction. There is an exception to this generalization for the case in which an enzyme equally stabilizes the groundstate and transition state, but catalysis can only occur in this situation if the enzyme is working below but not above saturation. There is a limit to this type of catalysis because if the enzyme binds the transition state and groundstate very tightly, the concentrations of enzyme and substrate required to maintain non-saturation conditions will be decreased. These concentrations could be so low that enzyme catalysis may not be observed. If a non-reacting substituent of a specific substrate contributes a large amount of binding energy, it is essential that this is not expressed in the groundstate or intermediate states in order to avoid saturation conditions and the low concentrations of enzyme and substrate required to observe non-saturation.

Maximum catalytic efficiency may be achieved by

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the enzyme stabilizing all transition states, but not intermediate states, in the pathway between reactants and products. It is obvious that an efficient enzyme must stabilize the transition state(s) of a reaction, but it is equally important that the enzyme does not excessively stabilize any intermediate. Stable enzyme intermediate states will bring about saturation conditions at a low concentration of substrate and valuable enzyme will be then tied up in an energy well (Jencks 1975; Page 1984; Knowles 1987; Burbaum *et al.* 1989).

For a given amount of binding energy between the substrate and enzyme the most effective catalysis will be obtained if this energy is used to stabilize the transition state, which maximizes the value of k_{cat}/K_m. For a given free energy of activation, and for a given substrate concentration, the maximum rate is obtained if the substrate is bound weakly, i.e. shows a high K_m. A low value of K_m, i.e. strong binding of the substrate or intermediate state, mediates against catalysis. In agreement with these ideas, the physiological concentrations of most substrates are below their K_m values (Fersht 1974).

A major question in understanding the discrimination between substrates is how does the system allow the binding energy between the 'non-reacting' parts of the enzyme and substrate to be expressed in the transition state and not the groundstate? If a 'non-specific' and a 'specific' substrate have identical reaction centres, the intrinsic binding energy between the reacting groups undergoing electron density changes and the enzyme should be similar. Differential binding of the transition states around the reaction centre is easy to visualize. Recognition of the geometrical and electronic differences between groundstates and transition states depends on suitably placed amino acids in the enzyme. How can the binding energy of the non-reacting part of the specific substrate stabilize the transition state but not overstabilize the enzyme–substrate or enzyme–intermediate complex? This is very relevant to building in recognition sites in enzyme inhibitors because it is often the binding energy between the non-reacting part of the substrate that accounts for catalysis. The binding energy could be used to 'destabilize' the substrate or to compensate for thermodynamically unfavourable processes necessary for reaction to occur. The importance of this interaction is shown by the observation that the observed binding constant (usually K_m values) of a specific substrate is often apparently 'weaker' or no 'tighter' than that for a non-specific substrate.

The preceding statements should not be interpreted too literally. That 'the enzyme stabilizes the transition state and destabilizes the groundstate' should not encourage us to believe that enzymes are there in a fixed state doing 'something' to the substrate. Stabilization is a mutual process – one could equally well describe the substrate in the transition state stabilizing the enzyme. The important change is the *difference* in energy between the groundstate – enzyme and substrate in their own environment – and the transition state of the enzyme–substrate complex. For example, the statement that 'a positive charge on the enzyme stabilizes the negative charge on the substrate' could easily be inverted. Furthermore, a positive charge on an enzyme would probably be 'neutralized' in some way in the groundstate of the isolated enzyme, for example, by anionic charges on a neighbouring part of the protein chain or buffers, and by solvation provided by both the rest of the enzyme and water. The rate of reaction would be controlled by the *difference* in free energy provided by these stabilizations in the groundstate and the transition state.

Although enzymes are often considered to be anthropomorphic and have found out how 'to get something for nothing', this is a doubtful achievement even for evolutionarily perfect enzymes. Enzymes cannot solvate and distort substrates with no energetic loss to themselves. The molecular recognition between enzymes and ligands depends on the *difference* between interactions of the isolated molecules and their environment, and the binding-energy interactions between the enzyme and ligand in the complex. Catalysis by enzymes depends on the *difference* between interactions in the transition state and those in the initial state of the isolated molecules (Jencks 1975; Page 1984, 1988). Very rarely will an interaction take place in one state that is completely absent in the other.

The interaction between the substrate S and its environment X (which is usually the solvent) has a complementary interaction in the enzyme E and its environment Y (which may also be the solvent or another site within the enzyme). The changes in interactions that occur when a complex is formed between E and S include those generated in the pair X and Y

$$S\cdots X + E\cdots Y \rightleftharpoons E\cdots S + X\cdots Y. \qquad (1)$$

The net change in the overall energetics depends on the difference in the free energy resulting from this interchange of the 'bonding' partners. The free-energy change reflects the difference in enthalpy and entropy of these interactions. For example, S may have a proton donor site that forms a hydrogen bond with the solvent water acting as an acceptor, X, which is replaced with a similar acceptor site in the enzyme. Usually, in the initial state, this same site in the enzyme will be hydrogen-bonded to water, and $X\cdots Y$ of equation (1) would correspond to the formation of a new hydrogen bond between water molecules, which accompanies enzyme–substrate complex formation (Page 1988; Fersht 1985).

At the reaction centre, electron density and geometrical changes in the substrate, on going to the transition state, may be stabilized by complementary charges and shape of the active site in the enzyme. These electron-density changes could be accompanied by a conformational change so that a large non-reacting group not bound to the enzyme in the groundstate becomes bound in the transition state. In general, however, it is less easy to see how the binding energy of the non-reactive part of the substrate is prevented from being fully expressed in the ES complex unless it is used to compensate for unfavourable processes. If the environment provided by the enzyme is unfavourable to the groundstate structure of the substrate, the binding energy from the

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non-reacting part of the substrate could be used to force the substrate into this 'unwelcoming hole'. Examples are: (i) a 'rigid' enzyme that has an active site complementary in shape to the transition state but not the groundstate; (ii) an active site that is non-polar and conducive to stabilizing a neutral transition state but destabilizing a charged substrate; (iii) desolvation or solvation changes of groups on the substrate or enzyme; and (iv) an active site where electrostatic charges on substrate and enzyme are similar.

If the binding energy is used to compensate for the induction of 'strain' in the substrate, it is essential that the 'strain' is relieved in the transition state to increase the rate of reaction. This would be the case if the changes in geometry of the substrate were in the direction that accompanies the reaction mechanism. The observed binding energy would be what is 'left over' after the strain has been 'paid for' and may thus appear to be weak. It is essential that the non-reacting group only exhibits its binding energy when the reactive centre of the substrate is bound to the active site. Alternative binding modes would otherwise result, leading to non-productive binding that does not affect k_{cat}/K_m and specificity, but does decrease K_m, leading to saturation conditions at lower concentrations of substrate. The binding energy of the substituent of the specific substrate may be used to prevent non-productive binding (Fersht 1985).

Amino acid side chains, not necessarily those near the active site, may have their exposure to water changed during catalysis. For example, a polar group may become exposed or protected from water on going from the groundstate to transition state, which would make a favourable and unfavourable contribution to the free energy of activation, respectively (Page 1984).

Probably the most important way that the binding energy is 'used' is to compensate for the unfavourable entropy change that accompanies formation of the ES and $ES^{\ddagger}$ complex (Page & Jencks 1971; Page 1977). The entropy loss that is required to reach the transition state may already have been partially or completely lost in the ES complex.

At 25 °C for a standard state of 1 M, the complete restriction of medium-sized substrates requires a decrease in entropy and an increase in energy of about 150 kJ mol^{-1}, an unfavourable factor of 10^8. This entropy change is that typically required to form a covalent bond in which the atoms are confined to a relatively small volume because of the loss of translational and rotational freedom (Page & Jencks 1971; Page 1973). If the enzyme-catalysed reaction requires the formation of a covalent bond then the binding energy of the non-reacting part of the substrate may compensate for this necessary but unfavourable entropy change. A non-specific substrate may have insufficient binding energy to compensate for the required entropy loss, resulting in a reduced value of k_{cat}/K_m. If the chemical mechanism of catalysis requires the involvement of other functional groups such as general acids or bases, metal ions or a change in solvation, then a further entropic advantage may be apparent. However, the contribution is smaller than that from covalent catalysis because the 'flexibility' of hydrogen bonds and metal ion coordination is greater than that for covalent bonds (Page 1984).

4. THE DIFFERENCE IN RATES DUE TO SOLVENT CHANGES

The use of binding energy to compensate for unfavourable solvation changes necessary for chemical reaction is a possible mechanism of catalysis because of the large solvation energies of groups and ions in water (Page 1984). Lone pairs which may act as general bases or nucleophiles will usually be 'solvated' by hydrogen bonding from either water or intramolecularly from the enzyme. Similarly, other potentially reactive groups will usually be 'charge neutralized' in the initial state of the enzyme. These reactive groups will normally require 'desolvation' before bond making or breaking can occur. This process is energetically expensive and yet an essential part of the normal activation energy, but may be compensated by favourable interactions between the substrate and enzyme. Attempts to elucidate the importance of solvation have traditionally been investigated by determining the effect of changing the solvent on the reaction rate. Again it is essential to appreciate that any changes in rate result from the *differences* in groundstate and transition state energies which the solvent may cause.

The parameter k_{cat}/K_m measures the free-energy difference between transition state $ES^{\ddagger}$ and groundstate E and S (equation 2). If γ_S,γ_E and $\gamma^{\ddagger}$ represent the activity coefficients of the substrate, enzyme and transition state, respectively, and are defined relative to a common value of unity in purely aqueous solution then k_{cat}/K_m for the enzyme-catalysed reaction in a given solvent mixture is related to its value $k^{E}_{cat}/K^{\circ}_{m}$, in pure aquesous solution by equation (3). For sparingly soluble substrates γ_S may be obtained from the solubilities, S and S°, measured respectively in the presence and absence of organic solvent (equation 4). The major difficulty is to estimate solvation effects on the enzyme because of the lack of a method for measuring γ_E. This can be overcome if solubility and kinetic data are obtained for two substrates S_1 and S_2. In a given solvent system it then becomes possible to eliminate γ_E because this is the same for both substrates. The effect of the solvent on the ratio of the two transition-state activity coefficients is given by equation (5). The right-hand side of equation (5) contains only measurable quantities and the transition state ratio for the two substrates S_1 and S_2, $\gamma^{\ddagger}_1/\gamma^{\ddagger}_2$ may be compared with the groundstate ratio γ_1/γ_2,

$$E + S \xrightarrow{k_{cat}/K_m} ES^{\ddagger} \qquad (2)$$

$$k_{cat}/K_m = (k^{\circ}_{cat}/K^{\circ}_{m})\,(\gamma_S\gamma_E/\gamma^{\ddagger}) \qquad (3)$$

$$\gamma_S = S^{\circ}/S \qquad (4)$$

$$\gamma^{\ddagger}_1/\gamma^{\ddagger}_2 = \frac{(k_{cat}/K_m)_2\,S_2(k^{\circ}_{cat}/K^{\circ}_{m})_1\,S^{\circ}_1}{(k_{cat}/K_m)_1\,S_1(k^{\circ}_{cat}/K^{\circ}_{m})_2\,S^{\circ}_2}. \qquad (5)$$

The rate of the α-chymotrypsin-catalysed hydrolysis

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of 4-nitrophenyl acetate and N-acetyl-L-trypotophan methyl ester in organic solvent mixtures decreases with increasing amounts of dioxane or propan-2-ol. Measurement of the solubilities of the substrates in the solvent mixtures show that the difference in reactivity of the two substrates with solvent composition is largely a groundstate effect (Bell *et al.* 1974). The organic solvent stabilizes the non-polar substrates relative to water. Changing solvents, or as in the next section, changing substituents, can change the energetics of a reaction by stabilizing or destabilizing the groundstate and these effects can be just as, if not more, important than those in the transition state.

5. THE DIFFERENCE IN RATES CAUSED BY CHANGING SUBSTITUENTS

The effects of changes in substrate structure on enzyme catalytic activity are often used to identify specific binding sites between parts of the enzyme and parts of the substrate. However, changes in reactivity are not necessarily caused by changes in binding interactions between the substrate and the enzyme. Changes in substrate structure can induce different intrinsic 'chemical' effects such as inductive, resonance and steric ones which may relatively stabilize or destabilize the reactant or transition states. Ideally, these effects should be separated from those intermolecular interactions between the substrate and the enzyme. One method of achieving this is to compare the relative rates of the enzyme-catalysed reaction, brought about by changes in substrate structure, with those of a mechanistically similar non-enzyme-catalysed reaction. It then becomes apparent that some changes in the reactivity of enzyme-catalysed reactions are due to intrinsic changes in substrate reactivity and are not caused by interactions with the enzyme.

β-Lactamases catalyse the hydrolysis of the β-lactam ring of penicillins (**1**) and other β-lactam antibiotics. The serine β-lactamases are thought to hydrolyse their substrates by the intermediate formation of an acyl-enzyme, similar to the process used by the serine proteases. However, unlike the latter, the nature of the general acid–base groups on the protein involved in proton transfer are not known. The pH-rate profile for k_{cat}/K_m for the serine β-lactamases shows a broad-shaped pH dependence with unknown ionizing groups of pK_a 5.6 and 8.6 (Page & Laws 1990; Buckwell & Page 1987).

β-Lactam antibiotics invariably have an anionic group, such as a carboxylate, at C3 in penicillins (**1**) or at C4 in cephalosporins (**2**). It is often assumed that this anionic group binds to lysine-234 in β-lactamase (Buckwell & Page 1987; Page & Laws 1990).

Changing the C4 carboxylate in cephalosporin to the corresponding lactone (**3**) actually increases the efficiency of the enzyme-catalysed reaction with k_{cat}/K_m increasing 54-fold. It is tempting to interpret this as evidence that the lactone (**3**) is a 'better' substrate than the cephalosporin (**2**) because of better recognition or binding. However, the lactone (**3**) is 130-fold more reactive towards hydroxide ion in its alkaline hydrolysis than is the corresponding cephalosporin (**2**). The increase k_{cat}/K_m for the lactone is therefore probably an intrinsic chemical effect within the substrate (Laws & Page 1989). However, the rate enhancement brought about by the enzyme is as great for the lactone (**3**) as it is for the cephalosporin (**2**). How can this be if the carboxylate group in the antibiotic is a primary recognition site? Either the latter is not the case or the lactone group contributes a similar binding energy to that of the carboxylate.

Interestingly, the pH-rate profile for the β-lactamase-catalysed hydrolysis of the lactone (**3**) shows a similar bell-shape to that for 'normal' substrates with the rate decreasing at high pH owing to an ionizing group of pK_a 8.6. Either this group does not correspond to a cationic one on the protein that binds the carboxylate or its deprotonation decreases the binding energy to both the carboxylate and the lactone groups. The latter explanation is conceivable because k_{cat}/K_m measures the difference between the reactant state structures and the transition state. Although it is reassuring to picture the mutual stabilization of a negatively charged carboxylate anion and a positively charged aminium ion, both groups will be almost certainly equally stabilized in the groundstate by solvent or complementary charged groups. The net binding energy represents the difference between these two states. Taking a carboxylate anion and an aminium ion out of their native environment and placing them close to each other may not therefore be energetically any more favourable than doing the same for a lactone. Although a lactone is not as polarized as a carboxylate anion, its solvation energy in the groundstate is correspondingly reduced. The difference in binding between the two systems may therefore be small. Alternatively, the carboxylate anion and the lactone may both *not* bind strongly to a recognition site on the enzyme, which could explain the difference in β-lactamase reactivities between cephalosporins and penicillins.

6. THE DIFFERENCE IN RATES DUE TO GEOMETRICAL FACTORS IN THE SUBSTRATE

It is of interest to compare the relative reactivity of substrates caused by geometrical differences, particularly those due to changes brought about by cyclization. For example, amides and peptides generally adopt the *trans*-Z-configuration (**4**) whereas small ring lactams of necessity adopt the *cis*-E-configuration (**5**). Peptidases and β-lactamases have some properties in common, for example, both groups have serine and zinc enzymes amongst their members (Buckwell & Page 1987; Page & Laws 1990). However, in general, peptidases do not catalyse the hydrolysis of lactams and lactamases do not catalyse the hydrolysis of peptides or amides.

Constraining β-lactams into bicyclic systems such as the penicillins (**1**) enforces even more differences between lactams and amides. Unlike the normally

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planar peptide bond, bicyclic β-lactams are butterfly shaped with the nitrogen being significantly out of the plane defined by its three substituents (Page 1987). The pyramidal configuration of nitrogen thus suggests its lone pair is predominantly on the α-exo and less sterically hindered side of the bridge structure (**1**) (Page 1987). Unlike planar peptides nitrogen inversion and rotation about the carbonyl carbon–nitrogen bond is not possible. It is often suggested that stereoelectronic effects (Deslongchamps 1975) dictate that nucleophilic attack upon amides should occur in such a direction that the nitrogen lone pair becomes antiperiplanar to the newly formed bond. This is unlikely to occur in penicillins as it would involve the nucleophile approaching from the sterically hindered β-endo face of the bicyclic system. In fact, in non-enzyme-catalysed reactions it has been shown that nucleophiles attack from the more favourable endo direction (Page 1987). If this process occurs in the β-lactamase-catalysed reaction the inference is that the configuration of the first formed tetrahedral intermediates are different for β-lactamases and peptidases, (**6**) and (**7**), respectively. Furthermore, as nitrogen inversion and carbon–nitrogen bond rotation cannot take place in (**6**), collapse of the tetrahedral intermediate will be facilitated by protonation on the nitrogen lone pair from the exo-face.

As described in the previous section the nature and location of the general acid–base catalytic groups assumed to be important for the serine β-lactamases are not known. It has, in fact, been suggested (Hertzberg & Moult 1987) that there are none and that the proton from the serine hydroxyl group is transferred directly to the β-lactam nitrogen. Chemical intuition, a dangerous concept, informs us that this is an unlikely process.

Another strange problem associated with β-lactams is that they do not undergo ring opening as readily as would be suggested by the strain energy of the four-membered ring (Page 1987; Webster *et al.* 1990). One possible explanation is that unlike acyclic systems in which carbon–nitrogen bonds are broken by a stretching motion, four-membered rings may be opened by rotation (**8**). A consequence of this would be that a proton donor on the enzyme which facilitates carbon–nitrogen bond cleavage would be situated in a different position than that required in the hydrolysis of acyclic peptides. Furthermore, substituents attached to the incipient amino group would move considerably upon ring opening the β-lactam. It would then be inappropriate to have the substrate's carboxylate tightly bound to the enzyme.

Serine β-lactamase catalyse the hydrolysis of both monocyclic and bicyclic β-lactams but not apparently acyclic amides and anilides. This is consistent with, but of course does not prove, the above hypothesis. It was mentioned in the previous section that binding the carboxylate anion of the β-lactam substrate may not be obligatory. Another relevant observation is that below pH 4 the β-lactamase-catalysed hydrolysis of substrates with carboxylate groups at C3 in penicillins and at C4 in cephalosporins becomes pH independent whereas for the other substituents k_{cat}/K_m carries on decreasing. This suggests that the undissociated carboxylic acid of the substrate is a good substrate for β-lactamase and is consistent with, but does not prove, the idea that there is not a rigid binding site for the carboxylate anion because this functionality is required to significantly move upon β-lactam ring opening.

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