



## TRANSITION STATE BOND ORDER AS A SOURCE OF MECHANISTIC VERSATILITY IN ENZYME CATALYSIS (\*)

*The intersecting-state model (ISM) is applied to the study of enzyme catalysis. It is shown that enzymes tend to maximize the transition state bond order,  $n^*$ . Such fact leads to quite fast and selective reaction processes. The ambident behaviour of some enzyme catalyses is discussed in terms of the control of reactivity by  $n^*$  and the reaction energy,  $\Delta G^\circ$ , which have opposite variations on  $\Delta G^*$  as a function of the polarity of the reaction sites. The intramolecular character of enzyme reactions has been found to be dominated by bond lengths and  $n^*$  parameters of the reactive bonds.*

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## INTRODUCTION

We have recently examined the origin of the catalytic power of enzymes [1], in terms of the intersecting-state model (ISM) [2]. This work has been aimed at developing a view of these catalyses that can be understood, at least in the first approximation, in relatively elementary terms, without resorting to elaborate quantum mechanical calculations. The main conclusion is that such a power is due to the concerted effect of several molecular factors, namely free-energy effects in the enzyme-substrate complexes, decrease in force constants and bond lengths of the reactive bonds and enhancements of the so called «transition state bond order».

ISM provides a minimum set of molecular parameters to interpret chemical reactivity for an unidimensional potential-energy model. The energy barrier is estimated through the intersection of two potential energy curves: one for the reactants and the other for the products. For a prototype reaction



the horizontal separation of the potential energy curves,  $d$ , corresponds to the sum of the bond extensions of BC and AB up to the transition state, as long as one can neglect the interaction between the electronic states at the crossing point of the potential energy curves. We have shown [2] that the sum of the bond extensions is given by

$$d = [(a' \ln 2/n^*) + (a'/2) (\Delta G^\circ/\lambda)^2] l \quad (2)$$

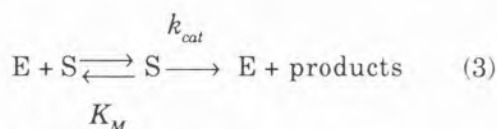
where  $n^*$  is the transition state bond order,  $\Delta G^\circ$  is the reaction energy,  $l = l_{AB} + l_{BC}$  the sum of the equilibrium bond lengths of reactant and product, and  $\lambda$  is a measure of the capacity of the transition states to accommodate energy;  $a'$  is a constant ( $a' = 0.156$ ).

In general, one uses harmonic potential energy curves, characterized by the corresponding force constants,  $f_i$ . Under this approximation the molecular parameters which control che-

mical reactivity can be classified into: i) reagent-product parameters: reaction energy ( $\Delta G^\circ$ ), force constants ( $f$ ) and bond lengths ( $l$ ); ii) transition-state parameters: transition state bond order ( $n^*$ ) and configuration mixing parameter ( $\lambda$ ). Although such a distinction is useful, one must realize that  $n^*$  is not independent of the electronic nature of reactants and products [2-5].

Since it generally takes more energy to stretch a bond than to change a bond angle, the emphasis on the control of chemical reactivity by the extensions of bonds rather than angles should arise no surprise. Even some rotational isomerizations have been interpreted within ISM [6] and only in some unimolecular isomerizations of small cyclic systems, together with bond extensions, there are significant contributions of the bending motions and bond angles changes along the reaction coordinate [7]. For the enzyme reactions we will only consider the bond extensions.

In terms of the current models of chemical reactivity, the concept of a variable bond order along the reaction coordinate is the least well known. Furthermore,  $n^*$  is the parameter which can produce the largest acceleration rate and one which is associated with the greatest number of mechanistic features. In view of these facts, in the present paper we intend to investigate further the importance of  $n^*$  in enzyme catalysis, for the hypothetical reaction



where  $k_{\text{cat}}$  is related to the reaction energy barrier by the Transition State equation

$$k_{\text{cat}} = (k_B T / h) \exp(-\Delta G_{\text{cat}}^* / RT) \quad (4)$$

Eq (2) shows that  $d$  is small when  $\lambda$  is high. This suggests that lower energy barriers are provided by floppy and disordered transition states ( $\Delta S^*$  high). Therefore, throughout the paper we will take  $\lambda \gg |\Delta G^\circ|$ .

## INTRAMOLECULARITY

Intramolecular reactions often proceed much faster than their intermolecular counterparts and this has been considered a source of catalytic activity [8]. Menger [9] has discussed in detail the origin of intramolecular acceleration, which can often amount to several powers of ten in magnitude, and has eliminated concentration effects, misalignment of reactants and entropic factors as the main source of such large rate enhancements. For example, entropies of activation correlate poorly with intramolecular efficiencies and this agrees with other findings on other chemical reactions; the intersecting-state model can reproduce well the experimental  $\Delta G^*$  values of several electron exchange reactions, but not the variations of  $\Delta H^*$  and  $T\Delta S^*$  [10].

ISM provides a good estimation of  $\Delta E^*$  for vapour phase reactions involving the breaking of one bond and the making of another one. However, for gas phase reactions of complex molecules or reactions in solution there are many other degrees of freedom than one stretching in reactants and in products. To keep the model unidimensional, one has to treat such degrees of freedom on a statistical basis by employing free-energies ( $G$ ) rather than internal energies ( $E$ ). Under such conditions ISM provides a good estimation of  $\Delta G^*$  for several types of reactions. In consequence,  $\Delta S^*$  does not show as an additional contribution for the reaction energy barrier\*, but is essentially a measure of the distribution of the internal energy in complex reacting systems.

Menger has suggested that the great source of the intramolecular rate acceleration is the «critical distance» of the reactants [9]. For example, for proton transfer reactions from  $\text{H}_3\text{O}^+$  to  $\text{H}_2\text{O}$  Schneider [11] finds by quantum mechanical calculation that decreasing the

\* When  $\Delta G^\circ \neq 0$ ,  $\Delta S^*$  can have an indirect effect on the energy barrier via the mixing entropy parameter when  $\lambda \ll |\Delta G^\circ|$ .

O-O distance from 2.95 Å to 2.55 Å increases the rates  $10^{11}$ -fold; the decrease from 2.75 Å to 2.55 Å increases the rates  $10^4$  times. Such decreases in distances between reactants are present in several intramolecular processes [9]. In enzyme reactions such effects have been invoked, for example, on the hydrolysis of thionoesters which are far less reactive than normal esters. This is attributed to the fact that the C=S bond length and the optimal S... HN= bond distances for the thionoesters are longer than the ester counterparts [12].

Within ISM such «distance effects» can be associated with bond lengths,  $l$ , due to changes in the equilibrium bond lengths of the bond-forming process in the products. For proton transfer reactions, with typical values of force constants  $f_r=f_p=f=4 \times 10^3 \text{ kJ mol}^{-1} \text{ Å}^{-2}$  and bond lengths  $l=2.0 \text{ Å}$  [4], one can verify the effect of the increase in  $l$  by ca. 0.4 Å, at a constant  $f$ . Virtually independent of  $\Delta G^\circ$  ( $\lambda \gg |\Delta G^\circ|$ ), the decrease in the rate constants is  $10^7$  times with  $n^*=0.5$  and ca. 30 times with  $n^*=1.0$ . This is the range of the transition state bond order values for the normal acids;  $n^*$  is close to 0.5 for carbon acids and to unity for HF (oxygen and nitrogen acids have  $n^* \approx 0.75\text{--}0.85$ ) [4]. Although the «distance effects» can produce large rate accelerations, particularly for the slow reactions ( $n^*$  low), the calculation suggests that other molecular factors are involved in the rate acceleration effect of 11 orders of magnitude; the most important is  $n^*$ .

#### EVIDENCE FOR THE IMPORTANCE OF $n^*$

Although changes in  $l$ ,  $f$  and  $\Delta G^\circ$  are all important in enzyme catalysis [1], changes in the transition state bond order are also certainly quite significant in some reactions. In principle  $n^*$  can be viewed as an empirical parameter of chemical reactivity, free from the effects of  $l$ ,  $f$ ,  $\Delta G^\circ$  and  $\lambda$ . However, for the majority of chemical reactions  $n^*$  has a simple physical meaning, given by counting the number of bonding, nonbonding and antibonding

electrons of the reactive bonds in the activated complexes.

For hydrogen transfer in hydride reactions, in the vapour phase, since one is dealing with the breaking and the forming of single bonds, the chemical bond order can be conserved along the reaction coordinate,  $n_{AB}+n_{BC}=1$ . In consequence, for the transition states at the thermoneutral situation  $n_{AB}^\# = n_{BC}^\# = 1/2$ . This situation is found when reagents and products do not possess nonbonding or antibonding electrons [2]. However, if there are occupied nonbonding or antibonding electrons in reactants and products, one of such pairs can acquire a bonding character when the transition states have a bent geometry [3-5]. This electronic siphoning increases  $n^*$  ( $n^*=1$  or  $3/2$ ), decreases  $d$  and, in consequence, diminishes  $\Delta G^\#$ .

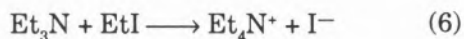
Solvents can also affect  $n^*$  through an interaction with the nonbonding pairs of electrons of reactants. For example, nucleophilic substitutions on methyl,



where X and Y are halogens, have  $n^*=1$  in the gas phase, independently of the nature of X and Y, due the conversion of a nonbonding pair of electrons from the reactants to a bonding pair in the transition state [13]. However, in liquid solutions  $n^*<1$ , approaching the limiting value of  $n^*=0.5$  for  $\text{X}=\text{Y}=\text{F}$ , in water. This limiting value implies that the total bond order is conserved along the reaction coordinate, because the nonbonding pair of electrons of the fluorine atom cannot acquire a bonding character at the transition state because of a strong interaction with the  $\text{H}_2\text{O}$  molecules. For other systems  $n^*$  depends on the nature of the solvent and of the nucleophile: i)  $n^*$  is higher with poor acceptor solvents (nonpolar) and lower with good acceptor solvents (polar); ii)  $n^*$  increases with the increase of the softness of the nucleophiles measured by their Mulliken electronegativities,  $I+A$  ( $I$  ionization energy,  $A$  affinity), and, in general,  $n^*$  is the

sum of the individual contributions of X and Y ( $n^* = C_n^\#(X) + C_n^\#(Y)$ ) [14].

Whereas reactions (5) are faster in nonpolar solvents, because their reactivities are dominated by  $n^*$ , the Menschutkin reaction



is faster in polar solvents, because its reactivity is controlled by the changes in  $\Delta G^\circ$  rather than  $n^*$  [13]. The development of charges favours a large decrease in  $\Delta G^\circ$  in the more polar media.

The transition state bond order can also be increased through the participation of electron-rich bonds ( $n=2$  or 3) in the reaction coordinate [7-15] and, in a less significant manner, through electronic siphoning of electron-rich substituents [7-16]. In contrast,  $n^*$  can be decreased through the loss of the synchronicity of the reactions, namely due to steric or solvent viscosity effects [16-18].

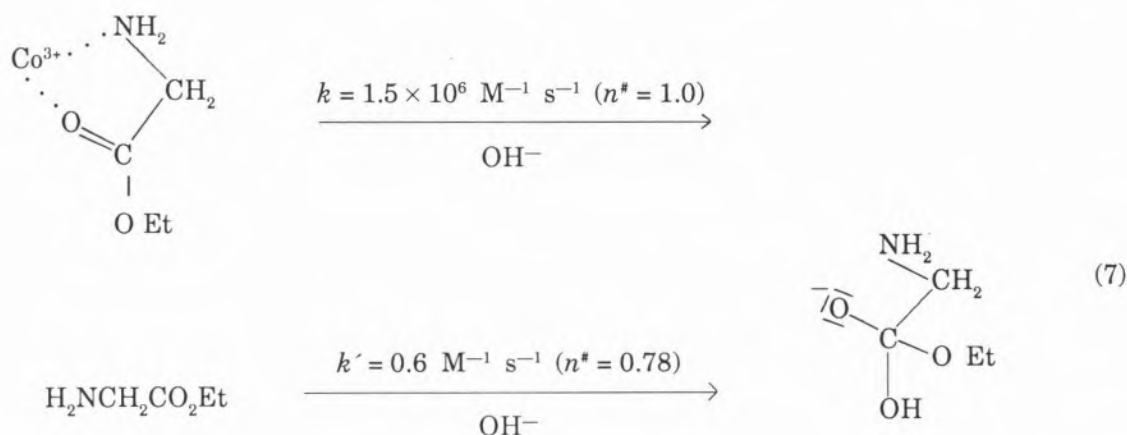
A strong evidence of the role of  $n^*$  in enzyme catalysis comes from mutagenesis studies, where mutant enzymes are prepared with changed aminoacid residues. Tyrosyl-tRNA synthetase catalyses the aminoacylation of tRNA; a change of histidine-45 by asparagine-45, which is positioned to form a hydrogen bond with the  $\gamma$ -phosphoryl group of ATP, does not affect the binding of ATP but increases the reaction barrier by ca. 19 kJ mol<sup>-1</sup> [19-20]. Since for such enzyme reactions we do

not expect a significant change in the force constants and equilibrium bond lengths of the reactive bonds, such variation in  $k_{\text{cat}}$  must be attributed to changes in  $n^*$  in the mutant enzyme.

There are also other enzyme reactions where changes in subsites increase  $k_{\text{cat}}$ , but do not change  $K_M$ . A good example is the structure of the active site of papain; the specificity for large hydrophobic residues in the  $S_2$  subsite is manifested in increased values of  $k_{\text{cat}}$  rather than in a tighter binding [20]. Within ISM such feature can be interpreted in terms of an increase of  $n^*$  in a less polar environment, at a constant  $\Delta G^\circ$ . The «charge relay system» [21] invoked in many enzyme reactions is a mechanism of siphoning electronic density into the transition states and, consequently, to increase  $n^*$ .

## CALCULATIONS FOR SELECTED ENZYME REACTIONS

One role for metals in metalloenzymes is that of a electrophilic catalyst, by «siphoning» electronic density into the transition states. This type of mechanism has been mimicked in model compounds; for example, the base catalyzed hydrolysis of glycine ethyl ether is increased more than 6 orders of magnitude when the compound is coordinated to  $\text{Co}^{3+}$  [22]

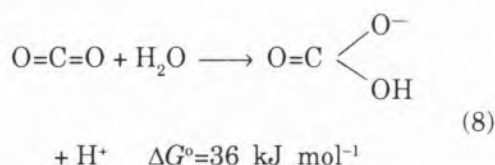




For the formation of the tetrahedral intermediate shown in reaction (7), the reactive bonds are C=O in reactants and two C–O bonds in the products (for two bonds  $f_p = \sqrt{2f_{C=O}}$ ). With the  $f$ ,  $l$  and bond strength data reported by Gordon and Ford [23], the relevant parameters are:  $f_r = 7.3 \times 10^3 \text{ kJ mol}^{-1} \text{ \AA}^{-2}$ ,  $f_p = 4.3 \times 10^3 \text{ kJ mol}^{-1} \text{ \AA}^{-2}$ ,  $l = l_{C=O} + l_{C-O} = 2.64 \text{ \AA}$  and  $\Delta G^0 = -30 \text{ kJ mol}^{-1}$ . This allows one to estimate the transition state bond orders presented above, as described elsewhere [1-5].

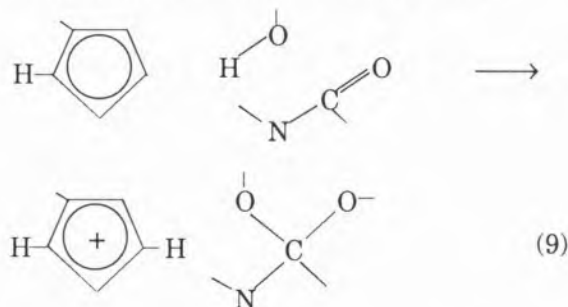
Apparently the metal ion siphons electronic density into the transition state, but the mechanism is possibly more subtle. If one of the oxygen lone pairs of the  $\text{CO}^-$  group becomes bonding in the transition state then  $n^* = 1.0$ . For the reaction in solution such pair interacts with the polar solvent molecules and  $n^* < 1.0$  ( $n^* = 0.78$ ). When the reagent is coordinated to  $\text{Co}^{3+}$ , the interaction of the  $\text{CO}^-$  group with the solvent molecules is prevented and the metal ion, behaving as a hard group, allows the  $\text{CO}^-$  lone pairs to become completely free to siphon electronic density into the transition state.

An interesting case of a very efficient metalloenzyme is carbonic anhydrase which catalyzes the reaction



The O enzyme is the most efficient, with  $k_{\text{cat}} = 10^6 \text{ s}^{-1}$  and  $\Delta G^0 = 40 \text{ kJ mol}^{-1}$  at room temperature. We will assume that the force constants and bond lengths of the reactive bonds have typical values [23]: reagents C=O,  $f_r = 7.3 \times 10^3 \text{ kJ mol}^{-1} \text{ \AA}^{-2}$  and  $l_r = 1.215 \text{ \AA}$ , and products (two C–O and one O–H bonds)  $f_p = \sqrt{\Sigma f_i^2} = 6 \times 10^3 \text{ kJ mol}^{-1} \text{ \AA}^{-2}$  and  $l_p = (2l_{C-O} + l_{OH})/3 = 1.269 \text{ \AA}$ . With this set of data the estimated transition state bond order is very high,  $n^* = 2.0$ . Not only does a lone pair of the  $\text{CO}^-$  group become bonding at the transition state, but electronic density is siphoned through a resonance mechanism *via* the C=O

bond. If the lone pair of the  $\text{CO}^-$  group was completely unable to become bonding at the transition state, then  $n^* = 1$  and  $\Delta G^0 = 79.5 \text{ kJ mol}^{-1}$ . This would correspond to a decrease in the reaction rate of ca. 7 orders of magnitude. Warshel and Sussman [24] have recently reported a calculation of the effect of a site-directed mutagenesis of rat trypsin, employing a simulation method based on a combination of the empirical valence bond (EVB) method and a free-energy perturbation method [25]. Here we would like to address the same problem within the much simpler ISM formalism. Craik *et al.* [26] have shown that the replacement of Gly-216 and Gly-226 by alanine produces a ca. 2000 fold reduction in the catalytic rate constant for amide hydrolysis, but a negligible change in  $K_M$  ( $< 20$ ). For the calculations we will assume a single kinetic step, which is probably not the real situation. This assumption implies a synchronous and concerted character for the reaction



In consequence the absolute  $n^*$  values should be smaller than the ones calculated. However, for comparative purposes such an effect can be neglected.

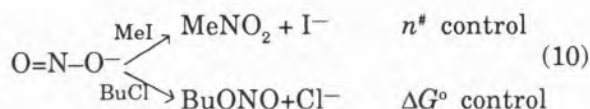
For reaction (9) the reactive bonds are: reactants, C=O and O–H, and products 2 C–O and a C–H bonds. Using the  $f$  and  $l$  data of ref. 23, one calculates  $f_r = 8.4 \times 10^3 \text{ kJ mol}^{-1} \text{ \AA}^{-2}$ ,  $f_p = 5.3 \times 10^3 \text{ kJ mol}^{-1} \text{ \AA}^{-2}$  and  $l = 2.4 \text{ \AA}$ ; the reaction energy is  $\Delta G^0 = 46 \text{ kJ mol}^{-1}$  [24]. To assess the effect of mutagenesis we will consider the effective  $n^*$  value as an weighted average of  $n_a^*$  of a carbon acid ( $n^* = 0.56$  [4]) and the  $n_h^*$  values for the hydrolysis reaction ( $n^* = (n_a^* + 2n_h^*)/3$ ). The maximum  $n_h^*$  is 1.0 and we will consider that mutagenesis causes geometric

changes that deform the oxyanion site in such a way that interaction of the  $\text{CO}^-$  lone pair with water molecules of the hydrogen network can occur. Obviously in simple terms it is not possible to translate the geometrical changes into changes of  $n_h^*$ . However, if one assumes that such value ranges between the values estimated previously for the hydrolysis of glycine ethyl ether in a polar environment and that coordinated to  $\text{Co}^{3+}$ ,  $n_h^*$  should range somewhere between 0.78 and 1.0. The effective  $n^*$  values will range between 0.85 ( $\Delta G^\circ = 104 \text{ kJ mol}^{-1}$ ) and 0.71 ( $\Delta G^\circ = 136 \text{ kJ mol}^{-1}$ ), i.e. mutagenesis should decrease  $k_{cat}$  by a factor smaller than  $4 \times 10^5$ . For a value of  $n_h^* = (1+0.78)/2$ ,  $n^* = 0.78$  the estimated decrease is 300 times which is in the range of the experimental effect, taking also into account the small variation of  $K_M$ .

Although the present explanation gives effects in fair agreement with experiment, we must point out that we have neglected any variation in the free-energy at the reaction site. However, since the reaction creates electrical charges,  $\Delta G$  can decrease strongly with an increase in the polarity of the reaction site. In consequence, if  $\Delta G^\circ$  dominates the effect of  $n^*$ , then a more polar environment would make the catalytic reaction faster, in contrast with the predictions based on the effect of  $n^*$ . Warshel appears to favour this last mechanism for the reactions under study [24,27].

## SELECTIVITY

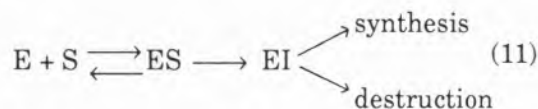
When one compares similar chemical reactions, the changes in reactivity are, in general, dominated by two structural factors:  $n^*$  and  $\Delta G^\circ$ . Under special circumstances one of those factors is the ruling one and interesting features of chemical reactivity can be observed. One of those features is ambident reactivity such as found in



The transition state bond order dominates the reactivity of one of the reactions and the reaction energy the other [28].

A similar situation can be present in the mechanism for editing in protein synthesis, as described by Fersht [29]. In the absence of an editing mechanism the errors in the amino-acid selection would be considerably larger than observed, for example, the isoleucyl-tRNA should favour isoleucine over valine by a factor of ca. 150, which combined with the higher concentration of valine *in vivo* systems would give an error rate ca. 1 in 30. Due to an editing mechanism the error is only 1 in 3000. The addition of tRNA<sup>Ile</sup> to the correct enzyme substrate complex gives Ile-tRNA<sup>Ile</sup>; the addition of tRNA<sup>Ile</sup> to the incorrect complex of valyl adenylate leads to hydrolysis in quantitative yields to valine and tRNA<sup>Ile</sup>.

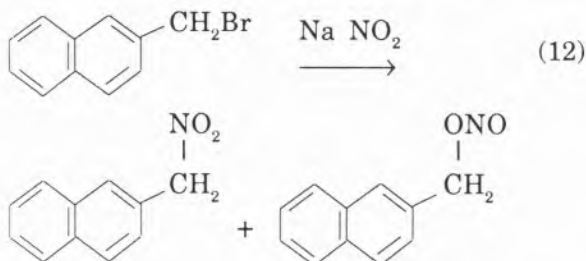
The overall mechanism involves high energy intermediates EI along the reaction path, which are then partitioned between further synthesis and destruction by hydrolytic editing.



The enzyme can operate such ambident behaviour in distinct and separate reaction sites [29], one more hydrophobic that enhances the  $n^*$ -control and diminishes the  $\Delta G^\circ$  effect, the other more hydrophilic that enhances the  $\Delta G^\circ$ -effect and diminishes the value of  $n^*$ . Synthesis is favoured in the correct intermediate by  $n^*$ -control in the hydrophobic site, and destruction via hydrolysis is favoured for the incorrect intermediate by  $\Delta G^\circ$ -control in the hydrophilic site. The enhancement of the ambident behaviour in proper sites allows a large ratio in the rate constants for the adequate reactions,  $k(\text{synthesis})\text{EI}^{\text{corr}}/k(\text{destruction})\text{EI}^{\text{corr}}$  and  $k(\text{destr.})\text{EI}^{\text{incorr}}/k(\text{synt.})\text{EI}^{\text{incorr}}$  which can amount typically to factors of  $10^2$ – $10^3$  [28].

Such behaviour conforms well with the qualitative features of enzyme analogue catalysis

by encapsulation of an organic substrate in the cavity of a macrocyclic compound. For example, the reaction of bromomethylnaphthalene with the ambident anion  $\text{NO}_2^-$



is catalysed by a macrocyclic-azacyclophane. The most remarkable feature of the reaction is the increase in the product ratio  $\text{R-NO}_2/\text{R-ONO}$  which corresponds exactly to the observed overall rate constant increase as a function of the catalyst-to-substrate concentration ratio [30]. The encapsulation of the reagents in a less polar environment allows the increase of  $n^\ddagger$  and increases the  $n^\ddagger$ -control product ( $\text{R-NO}_2$ ) ca. 4-5 times. In contrast, the opposite effect is observed upon the addition of open-chain alkylammonium salts during the substitution reactions [30]. The interaction of the lone pairs of  $\text{NO}_2^-$  with the added cation in a polar medium decreases  $n^\ddagger$  and  $\Delta G^\ddagger$  and favours the  $\Delta G^\ddagger$ -control product  $\text{R-ONO}$ .

A final word must be said about reaction selectivity in enzyme catalysis. There is an old principle in physical organic chemistry, the Reactivity-Selectivity Principle (RSP) [31], which states that the more reactive species tend to be less selective in their reactivity than the less reactive ones. Although enzymes have a substrate protection their optimization, which has been accomplished by nature over many million of years, should provide mechanisms for fast and selective catalysed reactions rather than fast and unselective processes. We have shown [5] that RSP is obeyed when the changes in reactivity are controlled by the changes in  $\Delta G^\ddagger$ ; in contrast an anti-RSP behaviour is found when the changes in reactivity are dominated by the

changes in  $n^\ddagger$ . This argument leads us to suggest a great importance of  $n^\ddagger$  in enzyme catalysis, because it ensures that the fastest reactions are also the most selective ones. Further, it appears that natural enzymes have evolved in the sense to attain the maximum  $n^\ddagger$  compatible with the molecular structure of the reacting partners.

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## RESUMO

### O papel da ordem de ligação no estado de transição na selectividade da catálise enzimática

O modelo de intersecção de estados (ISM) é aplicado ao estudo da catálise enzimática. Mostra-se que as enzimas tendem a maximizar a ordem de ligação do estado de transição,  $n^\ddagger$ , o que assegura a existência de reacções muito rápidas e selectivas. O carácter ambivalente de certas catálises é interpretado em termos do controlo da reactividade por  $n^\ddagger$  e pela energia da reacção,  $\Delta G^\circ$ , que conduzem a variações opostas na barreira de energia das reacções, em função da polaridade do meio. O efeito da intramolecularidade nas reacções enzimáticas é bem interpretado em termos dos parâmetros  $n^\ddagger$  e de comprimentos de ligação das ligações reactivas.