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The interpretation of site-directed mutagenesis experiments by linear free energy relations

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Fersht and co-workers have applied a linear free energy relation (Brønsted equation) to analyze site-directed mutagenesis experiments involving the enzyme tyrosyl-tRNA synthetase and have suggested that the Brønsted exponent is linearly correlated with the value of the reaction coordinate at the transition state. We point out that when the mutants differ solely through the formation or deletion of a hydrogen bond away from the reaction center, a linear free energy relation is expected only in limiting cases for which the Brønsted relation exponent is 0, 1 or ∞. The results may be correlated with a conformational coordinate but not with the development of the reaction coordinate *per se*.

Key words: Brønsted plots/site-directed mutagenesis/linear free energy relations

Introduction

Linear free energy relationships (LFER) have been widely used in physical organic chemistry to systematize the rates of similar reactions by relating them to the equilibrium constants (Hammett, 1940; Morrison and Boyd, 1973; Maskill, 1985). Most commonly LFER are phrased in terms of the Brønsted equation (Brønsted and Petersen, 1923) involving the exponent β,

$$k = (K_{eq}/K^{o}_{eq})^{\beta}\, k^{o} \qquad (1)$$

where K^{o}_{eq} and k^{o} are the equilibrium and rate constants for a reference reaction, while K_{eq} and k are the equilibrium and rate constants for a related reaction where a substituent has affected the rate and equilibrium but not the mechanism. In terms of the free energy of activation for the reaction, $G^{\ddagger}$, and the standard free energy difference between reactants and products, ΔG, equation (1) can be written

$$\Delta G^{\ddagger} = \beta \Delta\Delta G + \text{constant} \qquad (2)$$

Where $\Delta G^{\ddagger} = G^{\ddagger} - G^{\ddagger}_{o}$, $\Delta\Delta G = \Delta G - \Delta G_{o}$, and $G^{\ddagger}_{o}$ and ΔG_{o} are the activation and standard free energies for the reference reaction, respectively. Equation (2) assumes that dynamical factors are the same in the series of reactions. (If the rate constant is written $k = \varkappa k_{TST}$, k_{TST} being the transition state theory rate constant, then equation (1) is equivalent to assuming that the transmission coefficient $\varkappa$ is the same for all reactions in the series.) As commonly employed, reactions with the same mechanism are compared and if equation (2) holds ('linear' Brønsted plot), the exponent β is evaluated and used in relating the transition state and reactant or product states. Alternatively, the existence of a linear Brønsted plot has been used to argue that the same mechanism is involved for the members of the series.

General qualitative arguments for the relation between changes in reaction rates and equilibrium constants have been given for reactions in the gas phase (Agmon and Levine, 1980) and in solution (Marcus, 1968, 1969). For proton transfer reactions, in particular, Marcus has used a simple model to argue that the Brønsted exponent is a measure of the development of charge in the transition state, but not necessarily of the position of the transition state along the reaction coordinate (Marcus, 1968, 1969); for the latter, the intrinsic activation barrier, $G^{\ddagger}$ for $\Delta G = 0$, must be unchanged by the substitution. (Marcus restricted himself to the case where $G^{\ddagger}_{o}$ is the intrinsic barrier to reaction, which corresponds to $\Delta G = 0$. However, it is generally true for the case when $G^{\ddagger}_{o}$ is the barrier of the reference reaction corresponding to ΔG_{o}.) In spite of such limitations and experimental deviations (Jencks *et al.*, 1982; Pross and Shaik, 1983), the utility of linear free energy relations for correlating related reactions is unquestioned.

A number of applications of free energy relations to enzyme catalysis reactions have been made (Kirsch, 1972); a striking example is the recent work of Toney and Kirsch (1989) on the restoration of proton transfer of an inactive mutant of aspartate aminotransferase by the addition of a series of amines. Fersht and co-workers have employed LFER in the form of equation (1) to correlate the influence of mutations on the rate of catalysis of the formation of tyrosyl-AMP by the enzyme tyrosyl-tRNA synthetase (Fersht *et al.*, 1986a,b). The reaction considered by Fersht *et al.* proceeds with the binding of tyrosine (Tyr) to the enzyme active site (E) and the coordination of adenosine triphosphate (ATP) in position for the reaction,

$$E + Tyr + ATP \leftrightarrows E.Tyr.ATP \leftrightarrows E.Tyr - AMP.PP \leftrightarrows E.Tyr - AMP + PP$$

For this case, it is the rate enhancement of the reaction of the bound species that is important since the binding process is rapid. In fact, the increased binding to the enzyme of the transition state, relative to the reactants, appears to provide the dominant contribution to catalysis. The application of LFER to this system differs fundamentally from the standard situation in organic or biochemistry [see footnote 2 of Toney and Kirsch (1989)]. In the latter, small local structural changes coupled with electron redistribution along the reaction path are being considered. For the present case there are presumed to be larger structural changes and the interactions do not involve the part of the molecule participating directly in the reaction; i.e. there is presumed to be a conformational change in the Tyr.ATP complex in going to the transition (and product) states that alters interactions with the γ phosphate, ribose and base of the ATP, and the hydroxyl and amino groups of tyrosine, but not the α phosphate that is attacked by the carboxylate of the Tyr.

The applicability of LFER to tyrosyl-tRNA synthetase has been the subject of some discussions (Estel, 1987; Fersht, 1987) and it has been concluded that the experimental correlations are valid. Here we amplify the insightful analysis of Fersht *et al.* (1987) and point out that the linear Brønsted relation applies to the site-directed mutagenesis experiments on tyrosyl-tRNA synthetase because the exponent β is restricted to certain limiting values ($\beta = 0, 1, \infty$). Fersht *et al.* (1987) and Jencks (1985) have

suggested that observed linear free energy relationships often correspond to linear portions of a nonlinear curve.

Most of the mutations in the active site region examined by Fersht *et al.* (1986a,b, 1987) involve the deletion of a hydrogen bond to the substrate. Stabilizing groups are not adjacent to the reaction center, where charge develops during bond formation. As such, they monitor, primarily, the conformation of the substrate. Hydrogen bonding interactions are primarily electrostatic in character (Burley and Petsko, 1988) and the corresponding free energy varies nonlinearly with the hydrogen bond distance or orientation. Further, the charges on the donor and acceptor atoms of the substrate and protein residues, which form the hydrogen bonds, do not vary as the reaction proceeds. [If we model the hydrogen bond as either a dipole–dipole interaction, or a charge–charge interaction (Burley and Petsko, 1988), the free energy of the hydrogen bond will vary linearly with charge but nonlinearly as a function of the contact distance or orientation.] Because of the complex dependence of the hydrogen bond energy on distance, it seems very unlikely that a linear free energy relationship would result from interactions away from the reaction center that are due to hydrogen bonds; i.e. if they did vary in strength, there would be no reason to expect a linear correlation between the free energy changes $\Delta G^{\ddagger}$ and $\Delta\Delta G$. What is important in the cases studied by Fersht *et al.* is that the hydrogen bonds involved appear to be either present or absent as a function of substrate conformation and enzyme mutation. Further, any stresses induced in the protein as a result of hydrogen bond formation can be subsumed in the hydrogen bonding interaction. Thus, the experiments of Fersht *et al.* correspond to certain limiting cases of the Brønsted relation (see Figure 1). If a hydrogen bond is present in the reactant, transition and product states, the rate and equilibrium constant are unchanged by its absence (i.e. $\Delta G^{\ddagger}$ and $\Delta\Delta G$ are both zero, and the Brønsted relation does not apply) (Figure 1a); if a hydrogen bond is present only in the transition state (or in both the reactant and product states), $\beta \approx \infty$ (Figure 1b); if a hydrogen bond is present in the reactant and transition states (or only in the product state), $\beta \approx 0$ (Figure 1c); if a hydrogen bond is present in the transition and product states (or only in the reactant state), $\beta \approx 1$ (Figure 1d). In the application to an enzyme considered here, one is dealing with enzyme substrate, enzyme transition state and enzyme product complexes.

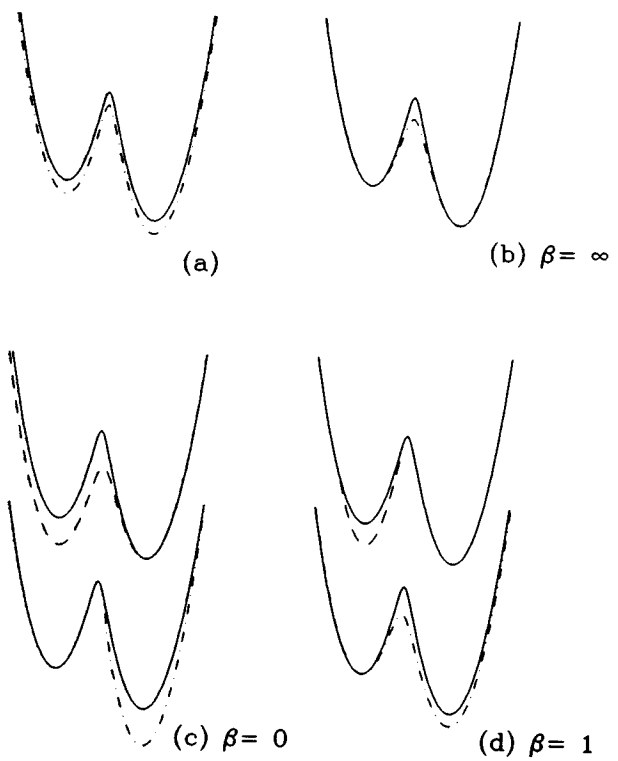

Fig. 1. Examples of potential energy surfaces leading to extreme values of the Brønsted exponent β: (**a**) equal stabilization of the reactant, transition-state and product; the Brønsted relation does not apply; (**b**) stabilization of the transition-state only; $\beta = \infty$; (**c**) equal stabilization of the reactant and transition state (upper), or equivalently, only the product (lower); $\beta = 0$; (**d**) stabilization of the reactant only (upper), or equivalently, equal stabilization of the transition-state and product (lower); $\beta = 1$.

In the deletion mutants studied by Fersht *et al.*, examples of several of the cases described above were found. The Tyr moiety of the substrate is hydrogen bonded to the same three residues (Tyr34, Tyr169 and Asp176) in the reactant, transition and product states; this corresponds to the uniform binding case of Albery and Knowles (1977). Thus, removal of this hydrogen bond has no effect on either the rate or the equilibrium constant (case a). Nevertheless, the interactions may be involved in binding the substrate sufficiently strongly to the active site. Sites Thr40 and His45 only affect the transition state and do not influence the reactant or product states (case b; $\beta \approx \infty$); both amino acids interact with the γ phosphate of ATP in a model of the transition state (Lowe and Tansley, 1984; Fersht *et al.*, 1987). Sites Cys35, His48 and Thr51 apparently can form hydrogen bonds to the base only in the transition and product states (case d; $\beta \approx 1$). The amino acids involved in cases (b) and (d) increase the rate of catalysis. No amino acid corresponding to case (c) was found; such a hydrogen bond would not accelerate the reaction.

The mutation of Thr51 to Gly, Ala or Pro is believed to involve loss of the same hydrogen bond between the OH of Thr and the ring oxygen of the ribose of ATP. However, each mutation affects the rate and equilibrium somewhat differently, indicating that additional steric interactions (e.g. van der Waals contacts) and possibly structural perturbations contribute to the observed results. Corresponding behavior is found in the series of mutants Thr40 → Ala, Gly and His45 → Glu, Asn, Ala, Gly.

The application of LFER to site-directed mutagenesis experiments on tyrosyl-tRNA synthetase appears to be useful as a means of ordering the experimental results. A value of $\beta \approx 0.8$, obtained by fitting the data for some of the mutants, is sufficiently close to the limiting exponent $\beta = 1$ for linear behavior to be observed over the available range. The result indicates that the stabilization energies of the transition and product states are similar (except for the unlikely case of only reactant stabilization) and that, therefore, the conformation of the transition state is similar to the product state with respect to the hydrogen bonds affected by these mutants. Since there are mutants with $\beta = \infty$, involving only hydrogen bonds to the transition state (except for the unlikely case of equal reactant and product destabilization), it is clear that there is a significant difference between the transition and product state; presumably it involves release of pyrophosphate because it is the γ phosphate of ATP that makes the hydrogen bonds affected by the mutants in the transition state.

When substitutions occur far from the reaction center, as in this case, there is no guarantee of a simple relation between the reaction coordinate and the Brønsted exponent. In other enzymes, there may well be examples of reactions where the side chains of active-site residues interact with the reaction center, whose charge develops during bond formation or cleavage. The

application of LFER to the analysis of mutants involving such residues can provide information about the detailed structure and electronic properties of the transition state. Additionally, in such systems it may be possible to employ the Hammett $\varrho\sigma$ relation (Hammett, 1935; Jaffé, 1953; Stock and Brown, 1968; Wells, 1963). This relation separates the effect of substituents on the rates of a series of related reactions into two parts; a substituent constant σ, which gives the change in equilibrium constant between the standard reaction in the absence and presence of the substituent at a given position (i.e. σ_i for substituent i with equilibrium constant K^i_{eq}) and a reaction constant ϱ, which determines the effect of various substituents on a given member of a family of a reaction, relative to those on the standard reaction and corresponds to the Brønsted exponent β. The Hammett relation can be written

$$k^i = k^o e^{\sigma_i \varrho}$$

where k^o is the rate constant of the standard reaction and k^i that of the reaction for which ϱ is the reaction constant; σ_i is the substituent constant, given by $\sigma_i = K^i_{eq}/K^o_{eq}$ where K^i_{eq} and K^o_{eq} are the corresponding equilibrium constants. For altered residues that stabilize or destabilize the formation of charge at the reaction center, it may be possible to correlate the stabilization between enzymatic reactions by assigning a substituent constant to individual amino acids. This is equivalent to assigning a specific free energy change to a particular mutation (e.g. Gly → Ala) which stabilizes or destabilizes a folded protein (Alber *et al.*, 1987).

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References

Agmon,N. and Levine,R.D. (1980) *Isr. J. Chem.*, **19**, 330–336.
Alber,T. *et al.* (1987) *Nature*, **330**, 41–46.
Albery,W.J. and Knowles,J.R. (1977) *Angew. Chem. Int. Engl. Ed.*, **16**, 285–293.
Brønsted,J.N. and Petersen,K. (1923) *Z. Phys. Chem.*, **108**, 185.
Burley,S.K. and Petsko,G.A. (1988) *Adv. Protein Chem.*, **39**, 125–189.
Estel,D.A. (1987) *Protein Engineer*, **1**, 441–442.
Fersht,A.R. (1987) *Protein Engineer*, **1**, 442–446.
Fersht,A.R., Leatherbarrow,R.J. and Wells,T.N.C. (1986a) *Nature*, **322**, 284–286.
Fersht,A.R., Leatherbarrow,R.J. and Wells,T.N.C. (1986b) *TIBS*, **11**, 321.
Fersht,A.R., Leatherbarrow,R.J. and Wells,T.N.C. (1987) *Biochemistry*, **26**, 6030–6038.
Hammett,L.P. (1935) *Chem. Rev.*, **17**, 125–136.
Hammett,L.P. (1940) *Physical Organic Chemistry*. McGraw-Hill, New York.
Jaffé,H.H. (1953) *Chem. Rev.*, **53**, 191–261.
Jencks,W.P. (1985) *Chem. Rev.*, **85**, 511–517.
Jencks,W.P., Brant,S.R., Gandler,J.R., Fendrich,G. and Nakamura,C. (1982) *J. Am. Chem. Soc.*, **104**, 7045–7051.
Kirsch,J.F. (1972) In Chapman,N.B. and Shorter,J. (eds), *Advances in Linear Free Energy Relationships*. Plenum, New York, Ch. 8.
Lowe,G. and Tansley,G. (1984) *Tetrahedron*, **40**, 113–117.
Marcus,R.A. (1968) *J. Phys. Chem.*, **72**, 891–899.
Marcus,R.A. (1969) *J. Am. Chem. Soc.*, **91**, 7224–7225.
Maskill,H. (1985) *The Physical Basis of Organic Chemistry*. Oxford University Press, Oxford, Ch. 10.
Morrison,R.T. and Boyd,B.N. (1973) *Organic Chemistry*. Allyn and Bacon, Boston.
Pross,A. and Shaik,S.S. (1983) *Acc. Chem. Res.*, **16**, 363–370.
Stock,L.M. and Brown,H.C. (1968) *Adv. Phys. Org. Chem.*, **1**, 35–154.
Toney,M.D. and Kirsch,J.F. (1989) *Science*, **243**, 1485–1488.
Wells,P.R. (1963) *Chem. Rev.*, **63**, 171–219.

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