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Linear Free Energy Relationships in Enzymes. Theoretical Analysis of the Reaction of Tyrosyl-tRNA Synthetase

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Abstract: Recent studies of genetically modified enzymes have indicated that changes in activation free energies, $\Delta\Delta g^{\ddagger}$, and changes in reaction free energies, $\Delta\Delta G_0$, are correlated by the relationship $\Delta\Delta g^{\ddagger} = \beta\Delta\Delta G_0$. The present work explores the basis for such linear free energy relationships (LFERs) in enzymatic reactions, focusing on the effects of mutations in tyrosyl-tRNA-synthetase (TTS). It is demonstrated that the optimal way to analyze LFERs is by describing the reaction in terms of pure valence bond (VB) resonance structures rather than in terms of partially formed bonds. The use of the pure VB representation allows one to evaluate the relevant LFER using Marcus-type concepts and to compare the predicted β to the observed one. Using a two-resonance-structure VB model for TTS produces $\beta \simeq 0.5$, which disagrees with the observed values of $\beta \simeq 0.83$ and $\beta \gg 1$ for two classes of mutations. Noting, however, that the phosphoryl transfer process in TTS has been described before as going through a high-energy intermediate, we describe this reaction in terms of three VB resonance structures. This accounts for the observed values of β and supports the validity of LFER in TTS. It is pointed out that LFERs are valid in proteins even when the changes in $\Delta g^{\ddagger}$ involve very anharmonic interactions like hydrogen bonds, since such relationships reflect the correlation between $\Delta\Delta g^{\ddagger}$ and $\Delta\Delta G_0$ rather than the correlation between $\Delta\Delta g^{\ddagger}$ and the effect of specific residues. However, obtaining LFER in proteins requires that the active site environment responds linearly to the change of charges during the reaction, and such a linear response is far from obvious. Fortunately, the simulation study presented in this work as well as previous simulations has demonstrated that active sites of proteins obey the linear response approximation. Such a behavior of highly anharmonic systems is due to the availability of many compensating polar interactions. This finding provides a theoretical basis for the experimental observation of LFER in TTS.

1. Introduction

Linear free energy relationships (LFERs) have been used extensively in physical organic chemistry to correlate reaction rates with the corresponding equilibrium constants.[1-3] Such relationships imply that the changes in activation free energies due to various perturbations, $\Delta\Delta g^{\ddagger}$, are correlated linearly with the concomitant change in free energy difference between the reactants and products, $\Delta\Delta G_0$, i.e.,

$$\Delta\Delta g^{\ddagger} = \beta\Delta\Delta G_0 \qquad (1)$$

The validity of this equation has been widely accepted because many reactions follow such relationships[1-3] and also because theoretical studies[4-7] have lent strong support for these relationships. Nevertheless, cases where LFERs seem to fail have also been documented,[7] and the existence of a linear correlation between $\Delta\Delta g^{\ddagger}$ and $\Delta\Delta G_0$ (for small $\Delta\Delta G_0$) is far from being

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universally accepted. In fact, the observed linear correlations have been viewed by some as examples of serendipity in organic chemistry and have been regarded as very useful, yet empirical, relationships. The validity of LFER is much less firmly established when applied to enzyme catalysis. Here the experimental[8-11] and theoretical[12-14] assertions of LFER are much more recent, and they are somewhat controversial. A case of particular interest is the catalytic reaction of tyrosyl-tRNA-synthetase (TTS), where a series of 10 active site mutants reproduce an LFER with $\beta \simeq 0.83 \pm 0.05$, while other mutants that are known to interact directly with the transition state of a reacting substrate gave a less unique LFER with $\beta \gg 1$. Despite this observation and theoretical simulations of Warshel and co-workers[12-14] that predicted regular LFERs in some enzymatic reactions, it has been argued by Straub and Karplus[15] that the TTS reaction should not display LFER with $\beta \simeq 0.83$, since the relevant interactions are not harmonic and are associated with sites that are far from the reacting atoms. This argument, however, has been disputed by Fersht and Wells.[16]

This work examines the theoretical basis for the observed LFER in TTS. It is shown that the observed values of β are consistent with a true LFER, provided that one considers the correlation between pure valence bond (VB) states, with fixed charge distributions, rather than the real reactant, product, and intermediate states.[6] The specific case of TTS is used to illustrate that the VB representation provides a general method to discuss LFER in proteins and to successfully predict the value of β in eq 1.

2. LFERs in Systems That Can Be Described by Two Pure Resonance Structures

Many reactions are characterized by transition states whose structural and electronic features are intermediate between corresponding features of the reactant and product states. Factors that stabilize the product state and thereby reduce the reaction free energy, ΔG_0, will also stabilize the transition state to some extent and will therefore reduce the activation free energy, Δg^*. Thus it is reasonable to expect a qualitative correlation of the form of eq 1, where β is a constant for small changes in ΔG_0.

While the above arguments are qualitative, they could be quantified by describing the reaction in terms of two VB resonance structures, one ascribed to the product and one to the reactant. These resonance structures (RSs) are kept in a pure VB representation, where the bonds are either fully made or broken and groups are either neutral or fully ionized. A good example is the S_N2 reaction depicted in Figure 1. The figure describes the free energy function associated with the potential surfaces V_1 and V_2 of two VB RSs as a function of a reaction coordinate that involves rotation of the solvent molecules and changes in the reacting fragments (the solute). The free energy functions which are defined rigorously in refs 5 and 6 reflect the probability that a system with the potential surface of a given RS will have a given value of the reaction coordinate. As can be seen from the figure, one can clearly expect a simple correlation between Δg^* and ΔG_0 for small changes of ΔG_0.

If the two free energy functions can really be described by two parabolae with equal curvature, then the dependence of the activation free energy on the equilibrium free energy conforms to a modified Marcus relationship:[6]

$$\Delta g^* \approx (\Delta G_0 + \lambda)^2/4\lambda - \bar{H}_{12}(x^*) + \bar{H}_{12}{}^2(x_0)/(\Delta G_0 + \lambda) \quad (2)$$

$$|\bar{H}_{12}(x_0)| < |\Delta G_0 + \lambda|/2$$

Here λ is the so-called "reorganization energy" which is definedin Figure 1, and we consider only the range $|\Delta G_0| < \lambda$. x_0 and x^* are defined in Figure 1, and H_{12} is the coupling term that mixes the two resonance structures, where $\bar{H}_{12}$ is the average value of this term at the given value of x. The first term of eq 1 is simply the well-known Marcus expression[4] for the nonadiabatic case with $H_{12}(x) \simeq 0$.

Once we assume (or prove) that eq 2 is valid, we can differentiate this equation and obtain the linear relationship

$$\Delta\Delta g^* \simeq \{(\Delta G_0 + \lambda)/2\lambda - \bar{H}_{12}{}^2/(\Delta G_0 + \lambda)^2\}\Delta\Delta G_0 = \beta\Delta\Delta G_0 \quad (3)$$

where the coefficient of $\Delta\Delta G_0$ is the β of eq 1. Although this β is a function rather than a constant, it has an almost constant value of $\beta \simeq 0.5$ for $|\Delta G_0| < \lambda$.

The LFER of eq 3 is a direct result of the assumption that the free energy functions are parabolae of equal curvature. Such an assumption is valid if the given system can be described by the linear response approximation, and recent simulation studies[18,19] have indicated that charge transfer reactions in solutions can

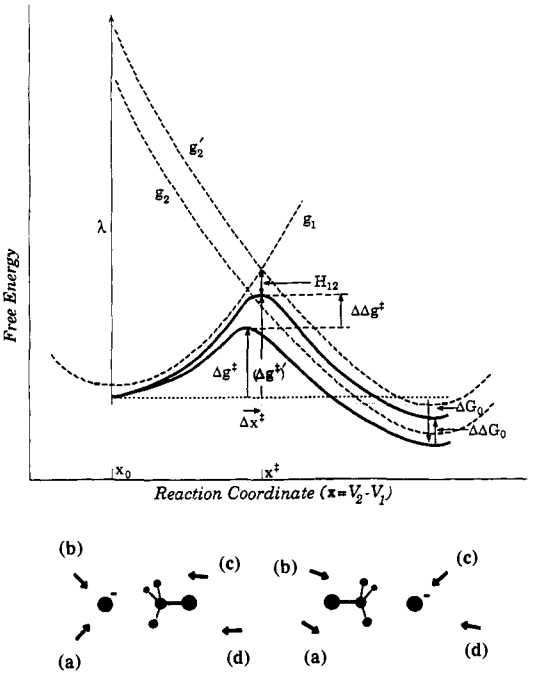

Figure 1. Schematic description of the relationship between the free energy difference, ΔG_0, and the activation free energy, Δg^*, in a two-RS model of an S_N2 reaction, where the bonding arrangement of state 1 and 2 is the same as the corresponding arrangement in the reactants and the products, respectively (as described in the lower part of the figure). The reaction coordinate, x, is taken as the difference between the potential energies V_2 and V_1 (see ref 6 for more details). The figure illustrates how a shift of the free energy function g_2 by $\Delta\Delta G_0$ (which changes g_2 to g_2' and ΔG_0 to $\Delta G_0 + \Delta\Delta G_0$) changes Δg^* by a similar amount. If, for example, we have four hydrogen bond donors (the dipoles in the lower part of the figure) and we mutate one of them (c) to a nonpolar molecule, we will find that ΔG_0 increases since state 2 is destabilized. As a result, we will find that Δg^* increases and the rate constant decreases.

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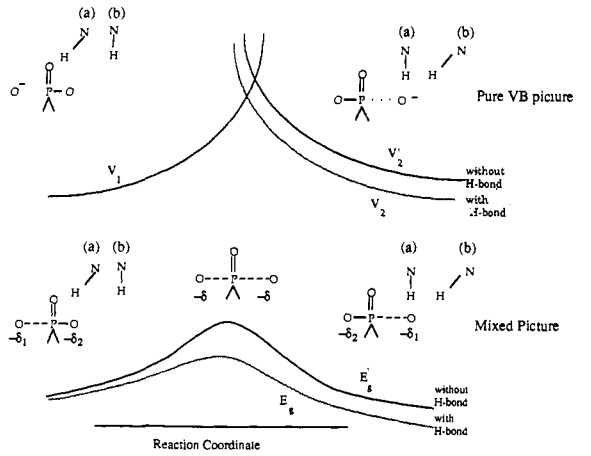

Figure 2. Illustration on the effect of an hydrogen bond on the pure and mixed VB representation. In this figure, we draw for simplicity the potential surfaces V_1 and V_2 rather than the corresponding free energy functions.

indeed be described by this approximation. Surprisingly, previous simulation studies[13,14] and the study which will be presented in section 3 have demonstrated that protein active sites can also be described by the linear response approximation. Thus, enzymatic reactions can be described by parabolae of equal curvature so that LFERs are also valid in such systems.

With the above discussion in mind, we may conclude that the $\Delta g^{\ddagger}$ and ΔG_0 of a reaction that can be described by two resonance structures will follow the LFER of eq 3. The resulting correlation between $\Delta\Delta g^{\ddagger}$ and $\Delta\Delta G_0$ is expected to follow eq 1, even if the actual interactions that change ΔG_0 are very anharmonic (in contrast to the argument of Straub and Karplus[15]). That is, as long as the change of a given interaction will not change the curvatures of the free energy functions and will only shift their minima, it will simply generate different values of ΔG_0, while leaving the correlation between $\Delta\Delta g^{\ddagger}$ and $\Delta\Delta G_0$ unchanged (the LFER reflects this correlation and not the correlation between $\Delta\Delta G_0$ and the hydrogen bond strength). For example, consider the hydrogen bonds that stabilize the second resonance structure in Figure 2. In this case, the dependence of ΔG_0 on the length of a given hydrogen bond might be highly nonlinear. A relatively short hydrogen bond to an atom that is negatively charged in a given resonance structure (e.g., hydrogen bond a in Figure 2) may contribute 5 kcal/mol, while another, longer bond (e.g., hydrogen bond b in Figure 2) would contribute only 1 kcal/mol. In such a case, one may assume that the effect of the deletion of hydrogen bonds cannot be described well by a reasonable LFER, since there would be no linear correlation between ΔG_0 and the hydrogen bond strength. However, LFERs correlate $\Delta\Delta g^{\ddagger}$ and $\Delta\Delta G_0$ (rather than $\Delta\Delta G_0$ and the hydrogen bond length), and the change of $\Delta G^{\ddagger}$ upon deletion of the first hydrogen bond will be around 2.5 kcal/mol (since $\beta = 0.5$ is predicted by eq 3), while the change upon deletion of the second hydrogen bond will be around 0.5 kcal/mol.

The fact that $\beta \simeq 0.5$ for $\Delta G_0 \simeq 0$ (which is obvious in the two-RS model) is not so easily recognized if one tries to describe LFER by considering the delocalized charges of the ground state obtained by mixing the two VB configurations (lower part of Figure 2). Such a mixed-state representation is clearly valid and can be related to the VB approach (see Chapter 1 in ref 6), but it makes it rather difficult to obtain a quantitative prediction of the changes in activation energy and geometry of the transition state as a result of changes in ΔG_0.

It is also important to point out that the β of eq 1 has not been taken as a unique result in traditional physical organic chemistry, perhaps because of the observation of deviations from this result and the fact that the linear response approximation could not be validated without microscopic simulations. It is argued here, however, that eq 3 is the *correct* result for a system that can be described by two resonance structures, and the deviations from this equation might indicate that the two-RS model cannot be used to represent the given system (see below).

With the above background, we may turn now to the observation[8] of $\beta \simeq 0.83$ in a series of mutants of TTS. In this case, ΔG_0 is much smaller than λ, so we expect to obtain $\beta = 0.5$ rather than $\beta = 0.83$. One could rationalize the deviation between the predicted and observed β values by suggesting different curvatures for the free energy functions of the two RSs. However, as already pointed out above, for aqueous solutions, the "solvent" contributions to the free energy functions of different VB structures appear to have similar curvatures,[18b] and preliminary studies (e.g., ref 14) also predicted similar curvature for free energy functions of proteins. As far as the intrinsic contributions by the reacting fragments (the solute) are concerned, it is still possible that the curvatures of the reactant and product free energy functions are different, but the difference is expected to be small in the case of TTS (see section 3). Furthermore, even the assumption of different curvatures or even changes in λ cannot account for the observation[8] of $\beta \gg 1$ for the class of mutations where a very large change in $\Delta g^{\ddagger}$ accompanies very small changes in ΔG_0.

Since the two-RS model predicts $\beta \sim 0.5$, which does not correspond to the experimental observations, we must consider a more appropriate description of the actual situation in TTS. The development of such a model is the subject of the next section.

3. LFER in a three-RS model of TTS

3.1. General Considerations. As argued above, the simple two-RS description gives a prediction of $\beta \sim 0.5$ that does not correspond with the observed β. Since it is hard to find conditions that would change this prediction, we must question the validity of the two-RSs model (rather than the LFER predicted by this model). Previous studies of TTS[9,10] have described the phosphoryl transfer reaction in terms of reactant and product and a high-energy pentacoordinate intermediate. The reaction in this model can be described in terms of the steps depicted in Figure 3. The pure VB description involves three resonance structures and two transition states (see Figure 4). In this case, the pentacoordinated transition state is considered as a real intermediate rather than a transition state. The transition states correspond to the intersections of the parabolae. The energy of the intermediate VB state (state 2) is hard to determine experimentally, but other crucial parameters such as $\Delta G_{1\rightarrow3}$ (the overall free energy of the reaction) and the largest of the two $\Delta g^{\ddagger}$ values (which is designated here as $\Delta g^{\ddagger}_{1\rightarrow3}$) are known from available experimental information.[8-10] If a true LFER exists, then we should find a correlation of the form

$$\Delta\Delta g^{\ddagger} = \Delta\Delta g^{\ddagger}{}_{1\rightarrow3} \simeq \beta\Delta\Delta G_{1\rightarrow3} \qquad (4)$$

To examine the free energy relationships that are expected from the model of Figure 4, it is useful to designate the minima of the free energy functions for the three RSs by the three

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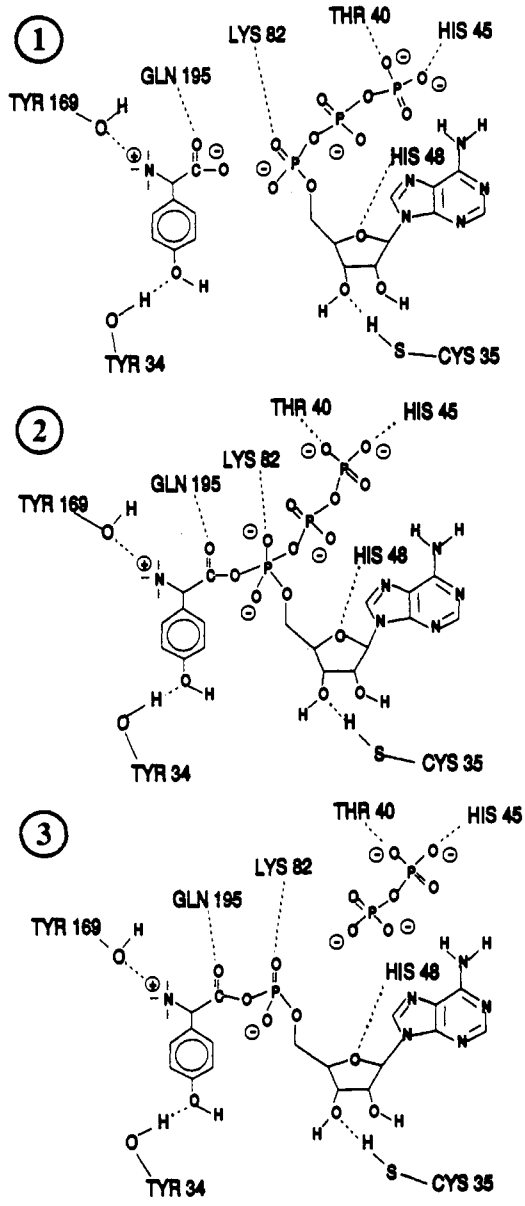

Figure 3. Three steps in the reaction of TTS.

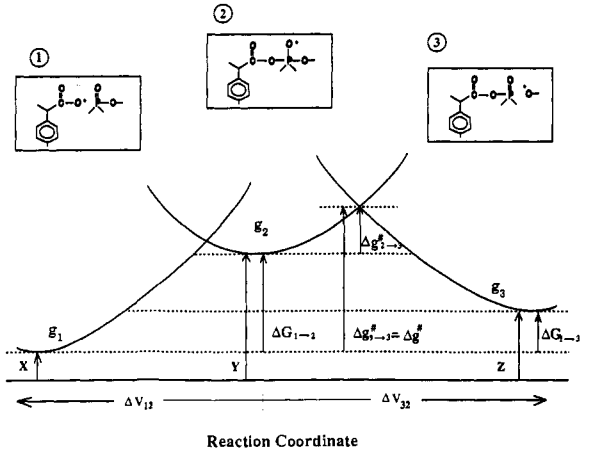

Figure 4. Schematic description of the three-RS model of TTS. The three resonance structures are depicted schematically on the upper part of the figure (see also Figure 3), and the corresponding configurations in the protein active site are drawn in the lower part of the figure.

independent variables X, Y and Z, respectively. With this notation and eq 2, we may write

$$\Delta g^{\ddagger}_{1\to3} \simeq \Delta G_{1\to2} + \Delta g^{\ddagger}_{2\to3}$$
$$= \Delta G_{1\to2} + (\Delta G_{2\to3} + \lambda_{23})^2/4\lambda_{23}$$
$$= (Y - X) + (Z - Y + \lambda_{23})^2/4\lambda_{23} \quad (5)$$

$$\Delta G_{1\to3} = (Z - X)$$

where we neglect the H_{12} terms since the effect of these terms on $\Delta\Delta g^{\ddagger}$ is small.

Now let us examine two limiting cases. The simplest one involves a mutation that changes only the product energy ($\Delta X = 0$, $\Delta Y = 0$, $\Delta Z = \theta_3$). In this case, it follows that

$$\Delta\Delta g^{\ddagger} = (\partial\Delta g^{\ddagger}/\partial X)\Delta X + (\partial\Delta g^{\ddagger}/\partial Y)\Delta Y + (\partial\Delta g^{\ddagger}/\partial Z)\Delta Z$$
$$= \theta_3\left(\frac{1}{2} - \frac{(Y - Z)}{2\lambda_{23}}\right) \quad (6)$$

$$\Delta\Delta G_{1\to3} = \theta_3$$

Thus, the linear correlation coefficient is $\beta = \Delta\Delta g^{\ddagger}/\Delta\Delta G_{1\to3} \simeq (0.5 - \Delta G_{3\to2}/2\lambda_{23}) \simeq 0.5$, while the experimentally determined coefficient is $\beta \simeq 0.83$. However, one has to take into account that a mutation in the active site of an enzyme is likely to have an effect not only on RS3 but also on RS2 and even on RS1 (where RSi designates the ith RS). Thus, we may write $\Delta X = \theta_1$, $\Delta Y = \theta_2$, $\Delta Z = \theta_3$ and define $\epsilon_1 = \theta_1/\theta_3$, $\epsilon_2 = \theta_2/\theta_3$. The resulting β is now given by

$$\beta \simeq \left[-\theta_1 + \theta_2 + (-\theta_2 + \theta_3)\left(\frac{1}{2} - \frac{(Y - Z)}{2\lambda_{23}}\right)\right]/[-\theta_1 + \theta_3)]$$
$$= \frac{\theta_3[-\epsilon_1 + \epsilon_2 + (1 - \epsilon_2)(1/2 - ((\Delta G_{3\to2})/2\lambda_{23}))]}{[\theta_3(1 - \epsilon_1)]}$$
$$\simeq \left[\frac{1}{2} + \frac{\epsilon_2}{2} - \epsilon_1\right]/(1 - \epsilon_1) \quad (7)$$

In this derivation, we assume that $\lambda_{23} \gg |\Delta G_{2\to3}|$, which is reasonable when $|\Delta G_{2\to3}| < \Delta g^{\ddagger}$ and $\Delta g^{\ddagger} < \lambda_{23}$, where $\Delta g^{\ddagger} \sim 15$ kcal/mol and $\lambda_{23} \simeq 50$ kcal/mol (see refs 5 and 14 for typical values of λ). Now, in the specific case of TTS, it appears that the substrate conformation in RS1 is quite different than that found in RS2 and RS3.[9] This was suspected on the basis of a comparison of the forward rate for the reaction and the k_{cat}/K_M values for the formation and destruction of tyrosyl adenylate on the enzyme.[24] Protein engineering experiments[8] have supported this speculation. In fact, it appears that almost all the mutations that give $\beta \simeq 0.83$ and change the energy of RS3 do not change the energy of RS1 (i.e., the binding of ATP), as the Michaelis complex is not affected by mutations that change the energy of the product. Thus we use $\epsilon_1 = 0$. With this selection, a value of $\epsilon_2 = 0.6$ (which implies that the effect of these mutations on state 2 is 60% of the corresponding effect on RS3) will reproduce $\beta = 0.8$. The same considerations can be applied to mutations that stabilize RS1 and RS2 but do not affect RS3.

The same arguments used to rationalize the observation of $\beta \simeq 0.8$ can be used now to account for the observation of $\beta \gg 1$ for mutations of residues that stabilize the intermediate RS. In doing so, we note that in the VB representation of Figure 4, we consider RS2 as an intermediate and locate the actual transition state at the intersection of the parabolae for RS2 and RS3, while the mixed state notation of ref 9 refers to RS2 as the transition state. In order to determine β for interactions that mainly stabilize RS2 (e.g., $\Delta Y \gg \Delta X \approx \Delta Z$ or $\theta_2 \gg \theta_1 \simeq \theta_3$), we can use eq 7 but now substituting $\epsilon_1 = \theta_1/\theta_2$ and $\epsilon_3 = \theta_3/\theta_2$. This procedure yields

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$$\beta \simeq \theta_2[-\epsilon_1 + 1 + (-1 + \epsilon_3)/2]/\theta_2[-\epsilon_1 + \epsilon_3]$$
$$= [1/2 - \epsilon_1 + \epsilon_3/2]/[\epsilon_3 - \epsilon_1] \quad (8)$$

Since ϵ_1 and ϵ_3 are small, we obtain here a very large β, and its actual value will depend largely on the uncorrelated difference between ϵ_3 and ϵ_1. Such an LFER is indeed observed experimentally for mutations of Thr40 and His45.[8] It should be pointed out that the above LFERs are merely elaborations of the basic Marcus idea to a three-RS system, and we have lost neither the predictive power nor the simplicity of the method.

3.2. Microscopic Simulations of LFER in TTS. Although the discussion in the previous section provides a feasible explanation for the observed LFERs in TTS, it does not prove that this reflects the actual energetics of the system. Thus it is important to try to establish our concepts by computer simulations which are based on a realistic model of the enzyme–substrate complex. Trying to reproduce the observed LFER in TTS is very challenging since the mutations affect different VB structures to varying degrees, and sometimes the changes are too small to be reproduced by current simulation strategies. However, it is much simpler to simulate LFERs that involve only two VB states (e.g., $\Delta g^{\ddagger}_{1\to2}$ vs $\Delta G_{1\to2}$ or $\Delta g^{\ddagger}_{3\to2}$ vs $\Delta G_{3\to2}$). Although such LFERs are not observed experimentally since the energy of the intermediate RS is too high, they provide the opportunity to examine the validity of the linear response approximation in TTS (see below).

Such simulations were performed using the previous described EVB method[5,6] and the program ENZYMIX.[21] The parameters used are similar to those used in related studies on the mechanism of staphylococcal nuclease[22] and rasvp21,[23] with the exception that we now consider three VB RSs as indicated in the upper part of Figure 4. The free energy perturbation (FEP) calculations involve 22 mapping steps, each with 2000 steps of 1 fs each. The simulations were carried out for a temperature of 300 K. The gas phase shift parameters (the $\alpha^{(i)}$ of ref 6) for RSs 1, 2, and 3 were taken as 0.0, −165.0 and −95.0 kcal/mol, respectively. These values were obtained by trying to achieve the best fit between the calculated and observed values of $\Delta g^{\ddagger}$ and $\Delta G_{1\to3}$ of the wild-type enzyme. This fit yields $\Delta G^{\ddagger}$ and $\Delta G_{1\to3}$ of 15.5 and 2.0 kcal/mol, respectively, where the corresponding observed values are 15.0 and 0.4 kcal/mol. It is important to note that the simulations involved the same intramolecular bonding potential functions for the two P–O bonds. Despite the fact that this assumption is a rough approximation, we found that the use of different Morse potentials accompanied by adjustment of the $\alpha^{(i)}$ to give the same $\Delta g^{\ddagger}_{1\to3}$ and $\Delta G_{1\to3}$ had little effect on our final results (the curvatures of the reactant and product states remained similar).

The results of the simulation for the His45Gly mutant are summarized in Figure 5. This calculation gives $\Delta\Delta g^{\ddagger} \simeq 4.0$ kcal/mol, as compared to the observed value of 3.5 kcal/mol. The corresponding calculated $\Delta\Delta G_{1\to3}$ is slightly less than 1 kcal/mol, while the observed value is 1 kcal/mol. This mutant has the smallest β and the largest $\Delta\Delta G_{1\to3}$ in the class of mutants that give $\beta \gg 1$. This is probably the reason why we obtained such good agreement between the calculated and observed values. However, as stated above, the reason for studying this mutant and also the Thr40Gly mutant is not the attempt to reproduce the observed β but the attempt to establish that the Δg curves in TTS are practically harmonic (see Figure 5). This point can be verified by treating varying $\alpha^{(3)}$ as a parameter and using it to change $\Delta\Delta G_{2\to3}$ (see ref 14). Such an examination, which is summarized in Figure 6, reproduces a very regular LFER between $\Delta\Delta g^{\ddagger}_{3\to2}$ and $\Delta\Delta G_{3\to2}$ with $\beta \simeq 1$ (note that this β cannot be evaluated from available experiments). This calculation establishes that the linear response approximation is indeed valid in TTS and that LFERs exist between the VB states of this system.

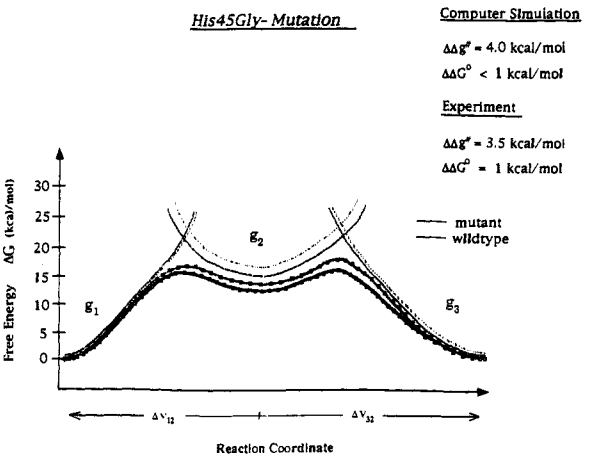

Figure 5. Result of the simulation of the His45Gly mutations (see upper right corner of the figure). As seen from the figure, the mutation mainly affects $\Delta g^{\ddagger}$ while leading to a substantially smaller change in $\Delta G_{1\to3}$.

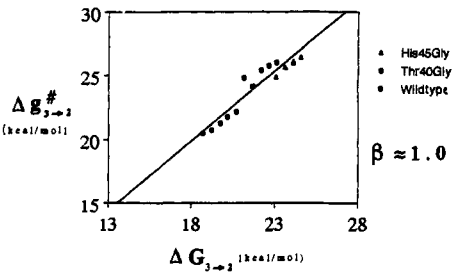

Figure 6. Calculated LFER obtained for the RS3 → RS2 process. The different points reflect the results of a parametric change of the gas phase shift (the $\alpha^{(3)}$), which represents an artificial change in the interaction between the active site and the substrate. The set of points for each mutant represents the results of small changes in $\alpha^{(3)}$ for this mutant.

Concluding Remarks

The present work explores the validity of LFERs in enzymatic reactions, focusing on the observed effects of mutations in tyrosyl-tRNA-synthetase. It is demonstrated that LFER can exist despite the possible anharmonicity and nonlinearity of the individual interactions involved in the different mutations. The overall correlation between $\Delta\Delta g^{\ddagger}$ and $\Delta\Delta G_0$ reflects the fact that a change in the free energy of the RSs is related in a simple way to the energy of the intersection of the free energy functions of these RSs (the transition state). The origin of this correlation would remain obscure without the use of a pure VB representation. Thus, while the change of ΔG_0 by a given mutation might be hard to predict (without expensive free energy perturbation calculations), the corresponding change of $\Delta g^{\ddagger}$ is predictable by a simple LFER. The only requirement for an LFER between resonance structures is that the mutations will not change the curvatures of the free energy functions or the corresponding λ values in a significant way. This has nothing to do with the nature of the interactions between the mutated residues and the substrate; different mutations might *shift* the free energy parabolae by a different magnitude, but this would only mean that such mutations will generate different $\Delta\Delta G_0$, while the correlations between $\Delta\Delta g^{\ddagger}$ and $\Delta\Delta G_0$ (the β) will remain constant.

The validity of the specific LFER of eq 7 requires that the entire active site rather than isolated residues will respond linearly to a change in the charges of the reacting system. This linear response requirement has been confirmed in simulation studies of several enzymes[12-22] and in the present work.

Our attempt to rationalize the LFER in TTS is based on the conclusion that a two-RS model with g of equal curvatures and $\Delta G_{1\to2} \simeq 0$ should give $\beta \simeq 0.5$, which does not correspond to the observed value of 0.83. One may still argue that the observation of $\beta \neq 0.5$ can be rationalized by assuming different

curvatures for the solute contributions to the g curves, having an H_{12} that changes in a different way for $x > x^{\ddagger}$ and $x < x^{\ddagger}$ or by a change of λ with mutations. These options cannot be excluded, but they cannot explain the observation of $\beta \gg 1$ for some mutations. Thus, it is essential to describe the reaction of TTS in terms of a three-RS model. Such a model allows us to rationalize the observed values of β for the two classes of mutations.

One of the main points of the present work is the observation that LFER should be formulated in terms of resonance structures rather than in terms of reactant and product states. Extending the analysis of LFER to this type of valence bond formalism appears to give a better explanation for the observed results and provides a more convenient method for analyzing reaction mechanisms. More importantly, the pure EVB description can be used to predict LFER, while the mixed representation cannot be used for this purpose. Note in this respect that computer simulations can be used to evaluate the free energy of different intermediates and to predict the corresponding LFER, even if such intermediates are hard to observe by regular experimental approaches. For example, this work reproduces a regular LFER with $\beta \simeq 1$ for $\Delta g^{\ddagger}_{3\rightarrow 2}$ and $\Delta G_{3\rightarrow 2}$. It would be extremely interesting to try to confirm this prediction by some clever experiment that measures directly the value of $\Delta G_{3\rightarrow 2}$.

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