Electrostatic Channeling of Substrates between Enzyme Active Sites: Comparison of Simulation and Experiment†

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ABSTRACT: Recent simulation work has indicated that channeling of charged substrates between the active sites of bifunctional enzymes or bienzyme complexes can be significantly enhanced by favorable interactions with the electrostatic field of the enzymes. The results of such simulations are expressed in terms of transfer efficiencies, which describe the probability that substrate leaving the active site of the first enzyme will reach the active site of the second enzyme before escaping out into bulk solution. The experimental indicators of channeling, on the other hand, are factors such as a decrease in the transient (lag) time for appearance of the final product of the coupled enzyme reaction or a decrease in the susceptibility of the overall reaction rate to the presence of competing enzymes or competitive inhibitors. The work reported here aims to establish a connection between the transfer efficiencies obtained from simulation, with the above-mentioned experimental observables. This is accomplished by extending previously reported analytical approaches to combine the simulated transfer efficiency with the Michaelis−Menten kinetic parameters K_m and V_{max} of the enzymes involved; expressions are derived to allow both transient times and steady state rates to be calculated. These results are applied to the two systems that have been studied both theoretically and experimentally. In the first case, that of the bifunctional enzyme dihydrofolate reductase−thymidylate synthase (DHFR−TS), the experimentally observed decrease in transient times is found to be consistent with a transfer efficiency of ≥80%. In the second case, that of a citrate synthase−malate dehydrogenase fusion protein, a transfer efficiency of 73% is consistent with the experimental transient time measurements. Separate and independent analysis of the effects of adding the competing enzyme aspartate aminotransferase gives a transfer efficiency of 69%, in excellent agreement with the transient time results. The transfer efficiencies thus obtained from experimental results are in both cases in good agreement with those obtained from simulations that include electrostatic interactions. One important discrepancy between simulation and experiment, is however, found in the reported effects of adding a competitive inhibitor in the DHFR−TS system: qualitatively different results are expected from the theoretical analysis. A possible reason for this apparent contradiction is discussed.

The term "substrate channeling" describes the idea that products of an enzyme-catalyzed reaction, which are required as substrates for reaction at a second enzyme, might be directly transferred (channeled) between the two enzymes without first escaping to bulk solution. The effect has been demonstrated in a number of settings, both with naturally occurring bifunctional enzymes (*1*, *2*) and with genetically engineered fusion proteins (*3*−*5*). The issue of whether any real advantage is to be gained from channeling in a physiological setting remains, on the other hand, highly controversial (*6*−*8*), although it is intuitively appealing to think that this would be the case.

Much of the published literature concerning substrate channeling preceded the development of any real atomic level understanding of the mechanism by which it might actually be achieved. The crystal structure of tryptophan synthase reported in 1988 (*9*) provided one view of how such a process might occur. In this case, the channeled substrate is indole, an uncharged nonpolar molecule. The enzyme prevents escape of the substrate by confining it to a 25 Å long tunnel running through the protein interior and connecting the two active sites; substrate diffuses along this tunnel to reach the second active site (the β-site). The structural development of the enzyme appears, however, to have extended beyond simply providing a conduit for the substrate since events at the two active sites are clearly coupled in such a way as to coordinate the entire catalytic process (for a review see ref 10).

The idea of a confining tunnel that prevents substrate escape by what are essentially steric means is perhaps the most obvious mechanism by which channeling could occur. A less obvious, but perhaps more general, mechanism was recently suggested by solution of the crystal structure of the bifunctional enzyme dihydrofolate reductase−thymidylate synthase (DHFR-TS;[1] *11*), an enzyme that has been shown to efficiently channel substrate between its active sites (*1*, *2*). In this case, the channeled substrate is dihydrofolate,

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[1] Abbreviations: CS, citrate synthase; MDH, malate dehydrogenase; PB, Poisson−Boltzmann; BD, Brownian dynamics; DHFR−TS, dihydrofolate reductase−thymidylate synthase; AAT, aspartate aminotransferase.

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which carries a formal charge of −2e, but in vivo, may contain additional γ-glutamate linkers, each carrying a charge of −1e (*12*). The two active sites are separated by ∼40 Å, and since there is no tunnel connecting the active sites, it is clear that the substrate must leave the protein interior and become solvent-exposed during the channeling process. Knighton et al. noted that the protein surface connecting the two active sites contains a number of positively charged residues; they therefore suggested that favorable electrostatic interactions between the negatively charged substrate and the positive electrostatic potential due to these basic surface residues might be the key factor in promoting efficient substrate channeling. Such a mechanism, if correct, would likely be of greater generality than that seen in tryptophan synthase for the simple reason that most metabolic intermediates are charged (*12*): tunnels are perhaps likely only to be found in the case of intermediates that are uncharged or which must be protected from exposure to water.

Support for the idea of an electrostatic basis for channeling has been provided by simulation work (*13*, *14*). The diffusion of small molecules in bulk solution and around enzymes is open to study through Brownian dynamics (BD) simulation techniques (*15*−*17*). In such an approach, the substrate is treated in a somewhat simplified fashion, and its motion is simulated under the influence of two types of forces, the first of which, always present, has a random component that models collisions with solvent molecules and results in the random, unguided diffusion which is characteristic of Brownian motion. The second type of force, if applicable, models the interaction of the substrate's charge with the electrostatic field of the enzyme. The magnitude of this second force is given by the product of the charge and the field, so if either of these terms is zero, the electrostatic force is zero and the substrate is only subject to the above-mentioned random force. It is the inclusion of both types of forces that lends BD simulations their importance, since they allow assessment of whether any electrostatic effects present are of sufficient magnitude to overcome the effects of random diffusion. In this regard it is worth noting that the observation of an apparently favorable electrostatic environment based on visual inspection does *not* necessarily give a reliable indication of its significance in determining substrate diffusion.

BD simulations of the type outlined above have been used previously to model substrate−enzyme encounter processes for enzymes that operate at or near the diffusion-controlled limit (for a review see ref 17): in such cases it is the rate at which substrate molecules arriving from bulk solution encounter the enzyme active site that is of interest. In contrast, our BD simulations of substrate channeling in bifunctional enzymes have been aimed at investigating the efficiency with which a substrate molecule starting at one active site transfers (channels) to a second active site. In the simulations the substrate interacts with the electrostatic field generated by the enzyme [obtained by numerically solving the Poisson−Boltzmann equation in and around the enzyme (see ref 18 for a review)] and simulation of its motion is continued until *either* it comes within a specified distance (6−8 Å) of the second active site, at which point it is considered to have been successfully transferred, or it attains a distance of separation (>90 Å) from the enzyme, sufficient for it to be considered to have escaped to bulk solution. Owing to the random nature of the diffusion process, simulations of substrate diffusion have to be repeated many times (typically ∼2000 times) to obtain a statistically meaningful sample of diffusion events.

The first system to which we applied the BD simulation technique was the DHFR−TS bifunctional enzyme discussed above (*13*), the structure of which was largely responsible for establishing the electrostatic channeling hypothesis. At zero ionic strength in the simulations, substrate was transferred from TS to DHFR with an efficiency of 95%; under the same conditions, an uncharged version of the same substrate was transferred with an efficiency of only ∼5%. The inclusion of electrostatic interactions between the substrate and the enzyme therefore had the effect of increasing the transfer (channeling) efficiency by ∼20-fold. As would be expected for an electrostatically dominated process, the efficiency was found to be strongly dependent on ionic strength: the increased shielding of electrostatic interactions that occurs at 150 mM was sufficient to reduce the transfer efficiency to ∼50%, which is still an order of magnitude more efficient than random diffusion. One additional finding from the simulation work was that substrate transfer in the reverse direction (i.e. from DHFR to TS) was almost equally efficient: at zero ionic strength the reverse transfer efficiency was ∼90%. This last result suggests that the channeling process in DHFR−TS is *not* directional: electrostatic interactions serve only to restrict the substrate's diffusion to the region connecting the two active sites; they do *not* serve to "pull" the substrate in the direction of the DHFR active site.

The observation of efficient channeling in these simulations prompted use of the same technique to investigate a role for electrostatic channeling in a second system (*14*), that of a fusion protein of citrate synthase and malate dehydrogenase (CS−MDH). Experimental characterization of this fusion protein (*5*) had suggested that the oxaloacetate produced by MDH was efficiently transferred to CS but had not revealed the mechanism by which this might be achieved. Application of the BD simulation technique to a modeled structure of this fusion protein gave a transfer efficiency of ∼45% at zero ionic strength when electrostatic interactions were included. This high transfer efficiency was obtained despite the active sites being separated by nearly 60 Å and not optimally oriented, aspects that are reflected in the fact that when electrostatic interactions were omitted, the transfer efficiency dropped to <1%.

Both simulation studies have therefore indicated that the inclusion of electrostatic interactions can result in an increase in substrate transfer efficiency of at least an order of magnitude. While these are encouraging results, it remains to be seen whether such increases are actually necessary to explain the experimental observations of efficient channeling in these systems. Until we establish a direct quantitative connection between our simulation observables and the experimental observables, we have no real way of knowing whether explanation of the experimental results *requires* electrostatic channeling or whether random diffusion (albeit at a much reduced efficiency) is sufficient. The establishment of this connection, and the comparison of the theoretical and experimental results that it allows, forms the basis of the work reported in this paper.

The expected kinetic behavior of coupled enzyme systems whether separated, statically associated, or dynamically associated has been the subject of a number of theoretical

treatments. Most such studies have focused on the transient time τ, which describes the duration of the lag phase preceding attainment of a steady state velocity in the coupled enzyme reaction. Conceptually, τ reflects the time required for the intermediate produced by the first enzyme to build up to a level necessary to maintain the steady state flux. For a separated two-enzyme system obeying pseudo-first-order kinetics (i.e. when the concentration of substrate is low enough that the steady state rate increases *linearly* with substrate concentration), it has been shown (*19*) that the expected transient time is given by $\tau = K_m/V_{max}$, where K_m and V_{max} refer respectively to the Michaelis constant and the maximum velocity of the *second* enzyme in the coupled system. More generally, i.e. when the enzymes obey Michaelis−Menten kinetics, the expression for τ becomes $K_m/(V_{max} - V_1)$ (*20*), where V_1 denotes the steady state rate of the first enzyme; it should be noted that use of this expression can lead to transient times considerably longer than those obtained under pseudo-first-order conditions.

The transient time is of interest in the present context because dramatic decreases in τ are one of the clearest indicators of the presence of substrate channeling. A theoretical treatment of channeling, and its predicted effects on τ, has been reported by Ovádi and co-workers under pseudo-first-order conditions (*21, 22*). For the case of statically associated enzymes, which is applicable to both DHFR−TS and CS−MDH, they derived the following expression: $\tau = \alpha\langle T_i\rangle + (1 - \alpha)\langle T_e\rangle$, where α corresponds to the channeling probability, while $\langle T_i\rangle$ and $\langle T_e\rangle$ refer respectively to the mean lifetimes of intermediate molecules that channel (i.e. remain "internal") and that escape to bulk solution before reacting (i.e. become "external"). To our knowledge, however, no extension of this method to the more general case of Michaelis−Menten kinetics has been reported.

Changes in the transient time are not the only means by which the presence of efficient substrate channeling may be deduced: a decreased susceptibility of the coupled enzyme system to the presence of competing (scavenging) enzymes or competitive inhibitors is also characteristic. An example of the former effect was provided in kinetics experiments carried out on the CS−MDH fusion protein (*5*). Increasing amounts of aspartate aminotransferase (AAT), which uses oxaloacetate as its substrate and therefore competes with CS for any present in bulk solution, had a dramatic effect on the activity of CS when a system of free (i.e. monofunctional) enzymes was used. When the same experiment was repeated with the fusion protein, AAT had much less of an effect, although some decrease in activity was still observed. This result was interpreted as showing that the oxaloacetate produced by MDH is effectively sequestered from bulk solution in the CS−MDH fusion protein and therefore cannot be easily scavenged by AAT.

An example of the effects of a competitive inhibitor was demonstrated in the kinetics studies of DHFR−TS reported by Trujillo et al. (*2*). The addition of increasing amounts of trimethoprim, which acts as a competitive inhibitor of DHFR (i.e. the second enzyme of the coupled system), resulted in a dramatic decrease in the overall rate when a system of free enzymes was used. Again, when the same experiments were performed with the bifunctional enzyme, the inhibitor had far less of an effect. These results were interpreted in terms of the *effective* concentration of the intermediate dihydrofolate being much higher in the bifunctional enzyme than in the free enzymes, allowing it to compete more effectively with the trimethoprim for binding at the DHFR active site.

$$S \xrightarrow{v_o} I \xrightarrow{K_{m,2};\, V_{max,2}} P$$

FIGURE 1: Basic reaction scheme describing the coupled enzyme reaction.

Any kinetic scheme that attempts to describe channeling and its effects on the behavior of a coupled enzyme system should clearly also be capable of incorporating these two competition effects. In the present paper we describe an extension of the analyses of Easterby (*19, 20*) and Ovádi et al. (*21*) to allow for both such effects and derive suitable expressions for the transient times and steady state rates. We then apply these expressions to the two experimental systems described above to allow comparison of the results of our computer simulations and the experimental observables. Note that in the description that follows we use the term "bienzyme complex" instead of "bifunctional enzyme" since the latter term is often applied rather loosely to enzymes that have a second noncatalytic role: the former term makes it clear that we are discussing a complex of two enzymes.

METHODS

(1) Effects of Substrate Channeling on the Kinetics of a Bienzyme Complex. We take as our starting point the approach adopted by Easterby (*19, 20*) for describing the transient time for a coupled system of free monofunctional enzymes. The reaction scheme we use is shown in Figure 1. We assume that the first enzyme operates at a constant velocity v_0 to generate the intermediate I, which is then subsequently converted to product P under the action of the second enzyme. We note that the velocity of the second enzyme must exceed that of the first if a steady state concentration of the intermediate is to be obtained. Using the condition for mass conservation, and assuming that the concentration of enzyme-bound intermediate is much lower than that of free intermediate (*20*), we can state

$$v_0 t = [\mathrm{P}] + [\mathrm{I}]$$

or

$$[\mathrm{P}] = v_0 t - [\mathrm{I}] = v_0(t - [\mathrm{I}]/v_0)$$

Following Easterby (*20*), we denote the steady state concentration of I as $[\mathrm{I}]_{ss}$ so that

$$[\mathrm{P}] = v_0(t - [\mathrm{I}]_{ss}/v_0)$$

A plot of [P] against t crosses the abscissa at $t = [\mathrm{I}]_{ss}/v_0$; this defines the transient time τ:

$$\tau = [\mathrm{I}]_{ss}/v_0 \qquad (1)$$

To calculate τ then, we simply need to know $[\mathrm{I}]_{ss}$. We now follow the approach outlined by Ovádi et al. (*21*) and partition the total population of I molecules into those which channel (I_i) and those which escape to bulk solution (I_e). We require that both subpopulations also be at a steady state, i.e.

$$d[\mathrm{I}_i]_{ss}/dt = d[\mathrm{I}_e]_{ss}/dt = 0$$

We reserve the term "channeling probability" for describing *only* the probability that the intermediate I is successfully transferred between the two active sites of the same bienzyme complex to form a Michaelis complex with enzyme 2: *we do not require that once this complex is formed a successful reaction follows*. Defined in this way, the channeling probability can be considered to be identical with that measured in our simulation studies. If the probability of channeling is denoted p_c, we can say immediately that the rate of formation of I_i is given by the product $p_c v_0$. The rate of disappearance of I_i is governed by the kinetic constants of the *second* enzyme, and we denote the rate of product formation as $k_{cat}[I_i]$ and the rate of dissociation of the Michaelis complex as $k_{-1}[I_i]$. Note that for this to be true we have to assume that binding of I_i to form the Michaelis complex is very much faster than either k_{cat} or k_{-1}: since the channeled diffusion of intermediates between active sites took of the order of only ~10 ns in our simulations (unpublished data), this is likely to be a reasonable assumption. The net rate of change of $[I_i]$ at steady state is therefore given by

$$d[I_i]_{ss}/dt = p_c v_0 - k_{cat}[I_i]_{ss} - k_{-1}[I_i]_{ss} = 0 \qquad (2)$$

This expression can be rearranged to give

$$[I_i]_{ss} = p_c v_0/(k_{cat} + k_{-1}) \qquad (3)$$

For the "external" intermediate I_e, the rate of formation is a sum of the rates associated with those molecules that do not channel [i.e. $(1 - p_c)v_0$] and those that do channel but do not proceed to form product [i.e. from above, $(k_{-1}[I_i]_{ss})$]. The rate of disappearance of I_e is given by the Michaelis−Menten rate (see for example ref 23), so the net rate of change of $[I_e]$ at steady state is

$$d[I_e]_{ss}/dt = (1 - p_c)v_0 + k_{-1}[I_i]_{ss} - \frac{V_{max,2}[I_e]_{ss}}{K_{m,2} + [I_e]_{ss}} = 0 \qquad (4)$$

which, using the above expression for $[I_i]_{ss}$ and rearranging, gives

$$[I_e]_{ss} = \frac{K_{m,2} v_0 (1 - p_c p_r)}{V_{max,2} - v_0(1 - p_c p_r)} \qquad (5)$$

Here p_r, which is given by $p_r = k_{cat}/(k_{cat} + k_{-1})$, denotes the probability that once a Michaelis complex is formed, it goes on to react to give products rather than dissociating to reform the intermediate. The term $p_c p_r$ therefore gives the combined probability that an intermediate molecule is both channeled *and* reacts. Now, since $[I]_{ss} = [I_i]_{ss} + [I_e]_{ss}$, combining eqs 3 and 5 we obtain

$$[I]_{ss} = \frac{p_c v_0}{k_{cat} + k_{-1}} + \frac{K_{m,2} v_0 (1 - p_c p_r)}{V_{max,2} - v_0(1 - p_c p_r)} \qquad (6)$$

and, therefore, from eq 1

$$\tau = \frac{p_c}{k_{cat} + k_{-1}} + \frac{K_{m,2}(1 - p_c p_r)}{V_{max,2} - v_0(1 - p_c p_r)} \qquad (7)$$

This result is similar in form to that quoted earlier from Ovádi et al. (*21*) but differs in allowing the second enzyme to obey Michaelis−Menten kinetics and in the introduction of the extra variable p_r, which does not appear in their analysis. The qualitative behavior to be expected of eq 7 becomes clearer when two reasonable assumptions are made. First, since k_{cat} and k_{-1} for enzymes typically range from 10 s^{-1} to much higher (*24*), the first term (≪1 s) will be much smaller than the second term (often ~10 s) and can therefore generally be neglected. Second, if we assume pseudo-first-order kinetics for the second enzyme (i.e. $[I_e] \ll K_{m,2}$ in eq 4), eq 7 simplifies to

$$\tau = \frac{K_{m,2}(1 - p_c p_r)}{V_{max,2}} \qquad (8)$$

From this, it can be seen that for any given value of p_r, τ is a linearly decreasing function of p_c. The presence of p_r, however, has an interesting consequence: the smaller p_r becomes, the less of an impact p_c has on decreasing the transient time. In other words, even if a particular bienzyme complex is capable of efficiently channeling its substrates from active site to active site (so that $p_c \rightarrow 1$), there will be *no* observable effect on the transient time of the coupled system if p_r is close to zero, i.e. if $k_{-1} \gg k_{cat}$. Enzymes that channel efficiently in vivo are therefore likely to have both p_c and p_r close to one.

From eq 2, the rate of product formation at steady state due to the channeling pathway is

$$\begin{aligned} d[P]/dt &= k_{cat}[I_i]_{ss} \\ &= p_c v_0 - k_{-1}[I_i]_{ss} \\ &= p_c v_0 - \frac{k_{-1}(p_c v_0)}{k_{cat} + k_{-1}} \\ &= p_c p_r v_0 \end{aligned} \qquad (9)$$

From eq 4, the rate due to $[I_e]_{ss}$ is

$$\begin{aligned} d[P]/dt &= \frac{V_{max,2}[I_e]_{ss}}{K_{m,2} + [I_e]_{ss}} \\ &= v_0 - p_c p_r v_0 \end{aligned} \qquad (10)$$

Adding these two rates of product formation together gives the expected result that channeling does *not* affect the overall steady state rate (*21*), which remains solely determined by v_0, the steady state rate of the first enzyme.

(2) Effects Resulting from Addition of a Third Enzyme That Competes for the Intermediate. We assume here that the presence of a competitor enzyme has no effect on intermediates that follow the channeling pathway but affects only those intermediates that escape to bulk solution prior to reaction at the second enzyme. With the addition of a third enzyme obeying Michaelis−Menten kinetics, the kinetic parameters of which are denoted $K_{m,3}$ and $V_{max,3}$, eq 4 becomes

$$d[I_e]_{ss}/dt = (1 - p_c)v_0 + k_{-1}[I_i]_{ss} - \frac{V_{max,2}[I_e]_{ss}}{K_{m,2} + [I_e]_{ss}} - \frac{V_{max,3}[I_e]_{ss}}{K_{m,3} + [I_e]_{ss}} \qquad (11)$$

which, when solved for $[I_e]_{ss}$, gives the following rather cumbersome expression for τ:

$$\tau = \frac{p_c}{k_{cat} + k_{-1}} + \{-K_{m,2}V_{max,3} - K_{m,3}V_{max,2} + v_0(1 - p_c p_r)(K_{m,2} + K_{m,3}) - \{[K_{m,2}V_{max,3} - K_{m,3}V_{max,2} + v_0(1 - p_c p_r)(K_{m,3} - K_{m,2})]^2 + 4K_{m,2}K_{m,3}V_{max,2}V_{max,3}\}^{1/2}\}/\{2v_0(V_{max,3} + V_{max,2} - v_0(1 - p_c p_r))\} \quad (12)$$

A much more manageable and easily understood expression is obtained when pseudo-first-order kinetics are assumed:

$$\tau = \frac{K_{m,2}K_{m,3}(1 - p_c p_r)}{V_{max,2}K_{m,3} + V_{max,3}K_{m,2}} \quad (13)$$

Note that for both expressions when the concentration of the third enzyme is zero, so that $V_{max,3}$ is zero, the original expressions for the transient time in the absence of any other competition effects (eqs 7 and 8) are returned. Also note that the transient time is predicted to *decrease* as the concentration of the third enzyme increases.

We can rearrange the expression for $d[I_e]_{ss}/dt$ (eq 11) to obtain an expression for the rate of formation of product due to this pathway. When this is added to the expression given earlier for the channeling pathway (eq 9), the following overall rate expression is obtained:

$$d[P]/dt = p_c p_r v_0 + \frac{V_{max,2}}{K_{m,2}}[I_e]_{ss} = p_c p_r v_0 + \frac{V_{max,2}}{K_{m,2}} \frac{v_0 K_{m,2}K_{m,3}(1 - p_c p_r)}{V_{max,2}K_{m,3} + V_{max,3}K_{m,2}} \quad (14)$$

From this result it is clear that as the concentration of the competitor enzyme increases, the contribution to the overall rate from the escape pathway decreases, while the contribution from the channeling pathway remains unchanged. This is therefore in line with the result that coupled enzyme systems that exhibit efficient channeling will be less affected by the presence of a competitor enzyme than those which do not.

(3) Effects Resulting from Addition of a Competitive Inhibitor for the Second Enzyme. Dealing with the presence of a competitive inhibitor for the second enzyme is considerably more complicated than the above case of a competing enzyme. The main difficulty lies *not* in treating the effects of the inhibitor on intermediates following the escape pathway, since steady state expressions can be straightforwardly derived for this case (see for example ref 23 for a derivation). Rather, the problems arise when one tries to decide to what extent the inhibitor affects intermediates that follow the channeling pathway. Two extreme scenarios can be imagined. The first is that the channeling probability p_c is totally unaffected by the presence of an inhibitor, which is akin to saying that channeled intermediates *always* displace any inhibitor present at the active site of the second enzyme. The second extreme scenario is that the channeling probability is scaled by the probability that the active site of the second enzyme is unoccupied by inhibitor; this is equivalent to assuming that channeled intermediates *never* displace inhibitor but instead are forced to escape to bulk solution if the site is already occupied by inhibitor. Obviously, neither case is completely realistic: it is more reasonable to assume that channeled intermediates will be capable of displacing inhibitor some, but not all, of the time and to an extent governed by the relative lifetimes of the intermediate in the vicinity of the enzyme and the inhibitor in the active site. Unfortunately, while we can estimate the latter from knowledge of the inhibition constant (K_I) of the inhibitor, we have no simple way of estimating the former. In what follows then, we simply replace p_c by p_c* to denote that the channeling probability is now some (as yet undefined) function of the inhibitor concentration, but we leave further consideration of this aspect for future work. With this change, the steady state concentration of channeling intermediates becomes, from eq 3

$$[I_i]_{ss} = \frac{p_c^* v_0}{k_{cat} + k_{-1}} \quad (15)$$

With regard to the escaped intermediates, the expression for $d[I_e]_{ss}/dt$ (eq 4) must be changed to account for the effects of competitive inhibition on the steady state rate of the second enzyme (see for example ref 23 for a derivation of this result):

$$d[I_e]_{ss}/dt = (1 - p_c^*)v_0 + k_{-1}[I_i]_{ss} - \frac{V_{max,2}[I_e]_{ss}}{K_{m,2}(1 + [Inh]/K_I) + [I_e]_{ss}} = 0 \quad (16)$$

In eq 16 [Inh] represents the concentration of the inhibitor and K_I denotes its inhibition constant. Making use of the expression for $[I_i]_{ss}$ given in eq 15 and rearranging gives

$$[I_e]_{ss} = \frac{K_{m,2}v_0(1 - p_c^* p_r)(1 + [Inh]/K_I)}{V_{max,2} - v_0(1 - p_c^* p_r)} \quad (17)$$

Note that this is essentially identical to the expression obtained earlier (eq 5) except for the addition of the multiplicative factor $(1 + [Inh]/K_I)$. This expression, when combined with that for $[I_i]_{ss}$ and used in eq 1, gives the following expression for the transient time in the presence of a competitive inhibitor:

$$\tau = \frac{p_c^*}{k_{cat} + k_{-1}} + \frac{K_{m,2}(1 - p_c^* p_r)(1 + [Inh]/K_I)}{V_{max,2} - v_0(1 - p_c^* p_r)} \quad (18)$$

When the channeling probability is zero, this expression reduces to that derived by Easterby (*20*) for free monofunctional enzymes in the presence of a competitive inhibitor. Again, assuming that the first term is negligible and that pseudo-first-order kinetics apply to the second enzyme, a somewhat simpler expression is obtained:

$$\tau = \frac{K_{m,2}(1 - p_c^* p_r)(1 + [Inh]/K_I)}{V_{max,2}} \quad (19)$$

Clearly, the addition of a competitive inhibitor has the effect of increasing the transient time, through increasing the concentration of the intermediate required to maintain a steady state rate. Again, as before, we can rearrange the expression for $d[I_e]_{ss}/dt$ to give the rate of product formation due to those intermediate molecules that escape to bulk solution. *We obtain an expression identical to that obtained earlier in the absence of inhibitor*. This leads to the

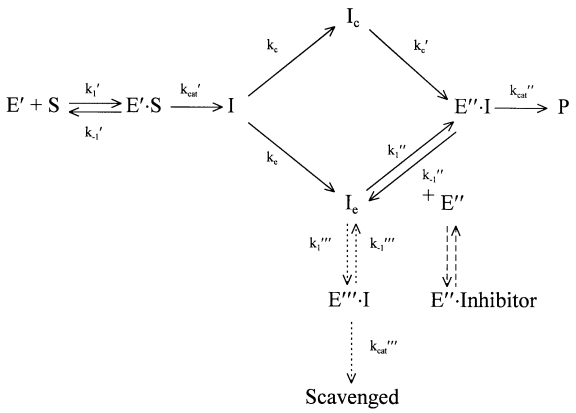

FIGURE 2: Reaction scheme used in numerical solution of differential equations.

important conclusion that in the coupled enzyme systems that we are considering here, the overall steady state rate is *not* affected by the presence of inhibitor; furthermore, this result is obtained whether or not $p_c \neq 0$. As we discuss in more detail under Results, this appears to be in conflict with reported experimental results.

(4) Numerical Solution of the Kinetics Equations. The above expressions are all analytical, but since they are derived under various assumptions, their range of applicability may in some cases not be completely clear. Perhaps the simplest way around this problem is to solve numerically the appropriate rate equations describing the time evolution of the system: such an approach is free of any assumptions, though it does not yield the conciseness of information given by an analytical solution.

The kinetic scheme that we have adopted is shown in Figure 2. In its most basic form it describes the conversion of substrate S to an intermediate I, catalyzed by a first enzyme E′, followed by conversion to products under the action of a second enzyme E″. Both enzymes are assumed to obey Michaelis−Menten kinetics, so reaction at each consists of two steps, a reversible binding step having rate constants denoted k_1 (for binding) and k_{-1} (for unbinding), followed by an irreversible catalytic step having a rate constant denoted k_{cat}. As in our analytical description, the scheme allows for two routes by which the intermediate arrives at the active site of E″: the upper route corresponds to channeling, whereby the intermediate remains associated with the bienzyme complex, while the lower route corresponds to escape of the intermediate to bulk solution. The partitioning of intermediates between the two pathways is performed respectively by the processes indicated by k_c and k_e. Conceptually, these rate constants can be thought of as deciding the fate of the intermediate: their relative values are set in such a way as to reflect the channeling probability p_c through use of the relationship $p_c = k_c/(k_c + k_e)$. In a sense then these rate constants are fictitious, since they do not correspond to any meaningful physical process. In practice, they are set to arbitrarily high (effectively infinite) values ($\sim 1 \times 10^{20}$ s^{-1}), so that the rate of the physical process of channeling is instead expressed by the second rate constant along the upper route, k_c'.

To apply this basic scheme, we need to know or be able to estimate values for each of the rate constants. k_1, k_{-1}, and k_{cat} are obtained from the Michaelis−Menten kinetic parameters of the enzymes, though it should be noted that

$$
\begin{aligned}
d[E']/dt &= -k_1'[E'][S] + k_{-1}'[E'\cdot S] + k_{cat}'[E'\cdot S] \\
d[E'']/dt &= -k_1''[E''][I_e] - k_c'[I_c] + k_{-1}''[E''\cdot I] \\
&\quad +k_{cat}''[E''\cdot I] \\
d[S]/dt &= -k_1'[E'][S] + k_{-1}'[E'\cdot S] \\
d[E'\cdot S]/dt &= k_1'[E'][S] - k_{-1}'[E'\cdot S] - k_{cat}'[E'\cdot S] \\
d[I]/dt &= k_{cat}'[E'\cdot S] - k_e[I] + k_c'[I_c] \\
d[I_c]/dt &= -k_c'[I_c] + k_c[I] \\
d[I_e]/dt &= k_1''[E''][I_e] - k_{-1}''[E''\cdot I] + k_e[I] \\
d[E''\cdot I]/dt &= k_1''[E''][I] - k_{-1}''[E''\cdot I] - k_{cat}''[E''\cdot I] \\
d[P]/dt &= k_{cat}''[E''\cdot I]
\end{aligned}
$$

FIGURE 3: First-order differential equations describing time evolution of the reaction scheme shown in Figure 2.

k_1 and k_{-1} are bound together by the relationship $K_m = (k_{cat} + k_{-1})/k_1$ (alternatively expressed as $k_{-1} = k_1K_m - k_{cat}$) and thus cannot be separated in any simple way. In describing the behavior of free enzymes, or a bienzyme complex that does not exhibit channeling (i.e. $p_c = 0$), this inability to separate k_1 from k_{-1} does not represent a problem, since the overall rate is determined only by k_{cat}, K_m, and the concentrations of enzyme and substrate. As was shown earlier, however (eq 7), in systems that do exhibit channeling (i.e. $p_c > 0$), this breakdown can be crucially important in determining whether channeling results in any observable effects since the relative values of k_{-1} and k_{cat} determine p_r through the relationship $p_r = k_{cat}/(k_{cat} + k_{-1})$. In practice, a range of physically plausible values of k_1 was investigated; for the most part, a value of 1×10^{10} M^{-1} s^{-1} was used.

As discussed above, the fictitious rate constants k_c and k_e were assigned effectively infinite rates, with their ratios being altered to reflect the channeling probability p_c. The rate constant k_c', which describes the actual rate of the channeling process, was estimated from the mean time of successful channeling events in our previous BD simulations (*13, 14*). In general, diffusion from the active site of the first enzyme to the active site of the second enzyme took ~10 ns; from this, we estimate a rate of 1/10 ns, i.e. 1×10^8 s^{-1}. In practice, this process is so much faster than the alternative route (escape to bulk solution followed by binding to a second bienzyme complex) that the results obtained are insensitive to the exact choice of k_c'. It should be noted that the actual channeling process is in this scheme considered to be irreversible. This might at first sight seem inconsistent with the results of our earlier BD simulations, since it was shown that in the case of DHFR−TS (*13*), transfer of substrate was equally efficient in the reverse direction, i.e. diffusing from DHFR to TS. It might appear then that our scheme should allow for the possibility that I escapes from the I·E″ complex and stays close to the bienzyme complex instead of escaping to bulk solution, prior to binding again to re-form the I·E″ complex. However, this process (which is independent of whether the initial I·E″ complex was formed from I_c or I_e) is already *implicitly* included in the kinetic parameters K_m and k_{cat}.

Given values for each of the rate constants shown, together with values of the initial concentrations of substrate S and the two enzymes, E′ and E″, the predicted time evolution of the system is obtained by solving the set of differential equations shown in Figure 3. This was done using FORTRAN routines taken from Numerical Recipes (*25*) designed for the purpose of integrating so-called "stiff" differential equations, of the type commonly encountered in the field of chemical kinetics.

Figure 2 also indicates two possible extensions of the basic scheme, to accommodate the presence of a competitive inhibitor of the second enzyme or a competing enzyme. In the case of the competitive inhibitor (dashed arrows in Figure 2), the ratio of the rate constants k_1 and k_{-1} for binding to E″ are obtained from the inhibition constant K_I, which is an experimentally obtainable quantity. In the case of the competing enzyme (dotted arrows in Figure 2), the new rate constants required are again those of an enzyme obeying Michaelis−Menten kinetics. The inclusion of either species necessitates additional differential equations to be included in the list given in Figure 3 but involves no new conceptual issues.

RESULTS

DHFR−TS: Transient Time Measurements. Kinetics experiments on the bifunctional DHFR−TS from Toxoplasma gondii and monofunctional analogues have been reported by Trujillo et al. (*2*). For the free monofunctional enzymes the observed transient times were in good agreement with those predicted by Easterby's expression ($\tau = K_m/V_{max}$; 19), a result that indicates that under their conditions pseudo-first-order kinetics are applicable. Accordingly, we use our pseudo-first-order expressions to analyze the results obtained for the bifunctional DHFR−TS. For three different sets of conditions, Trujillo et al. reported an undetectable transient time (i.e. $\tau < 10$ s) for formation of product in the coupled enzyme assay of the bifunctional enzyme. The ratio of the observed τ (<10 s) to that calculated by assuming no channeling (46 s) allows us to obtain $p_c p_r$, since $1 - p_c p_r \leq$ 10 s/46 s (eq 8); therefore, $p_c p_r \geq 0.78$. As noted by Trujillo et al. (*2*) then, at least 80% of dihydrofolate molecules are successfully channeled. We can combine this value for $p_c p_r$ with the maximum value for p_c (0.95) obtained in our BD simulations (*13*) and estimate that p_r must be ≥0.82; that is, at least four of every five Michaelis complexes formed at the DHFR active site subsequently react instead of simply dissociating.

DHFR−TS: Effects of Increasing Ionic Strength. Trujillo et al. (*2*) additionally conducted experiments at a NaCl concentration of 0.5 M as a preliminary test of the electrostatic channeling hypothesis: the inclusion of salt would be expected to reduce the strength of electrostatic interactions and, therefore, reduce the efficiency of channeling so as to produce an observable increase in the transient time. Under their conditions, and assuming that K_m of DHFR for dihydrofolate is *unchanged* by changing salt concentration, the expected transient time at 0.5 M NaCl with zero channeling assumed is $\tau_{calc} = 15.7$ s. Experimentally they obtained a value of $\tau_{exp} = 10$ s. This then means that $1 - p_c p_r = 10$ s/15.7 s, i.e. $p_c p_r = 0.36$. If we assume from before that $1.00 \geq p_r \geq 0.82$, then we obtain $0.44 \geq p_c \geq 0.36$. We previously reported simulation results for salt concentrations up to 0.25 M (*13*); we have since extended these simulations to 0.5 M and obtained the value $p_c = 0.25$, which is somewhat lower than the range of values expected. Considering that the transfer efficiency undergoes a 3-fold decrease on going from zero ionic strength to 0.5 M, and given the uncertainty in K_m at 0.5 M, our calculated value for p_c is in quite good agreement with the experimental result. It is worth pointing out that the increased activity observed experimentally for DHFR at the higher salt concentration (*2*) is consistent with a decreased K_m value, which would have the effect of *decreasing* the expected transient time: in such a situation the range of p_c values would be lower than stated above and thus would be expected to be closer to the value of 0.25 obtained in our simulations.

DHFR−TS: Effects of Added Competitive Inhibitor. Trujillo et al. (*2*) found that the activity of monofunctional DHFR in the coupled enzyme assay was strongly diminished by the addition of the competitive inhibitor trimethoprim [see Figure 5 of Trujillo et al. (*2*)]. When the bifunctional enzyme was used, activity was again reduced, but to a lesser extent: for a TMP concentration of 20 μM, the DHFR activity of the monofunctional DHFR was completely destroyed, while that for the bifunctional enzyme was only reduced to ≈75% of its value in the absence of TMP.

We have outlined under Methods the difficulties in treating the effects of competitive inhibition theoretically; we are forced to make the channeling probability dependent on the inhibitor concentration, although we have left the nature of this dependence undefined. Regardless of the concentration dependence of p_c*, however, the experimental findings are at odds with our theoretical analysis, since we found that the overall rate should be unaffected by the presence of a competitive inhibitor. To us, the most plausible explanation for the discrepancy is the following. While our analysis suggests that the steady state rate is not affected by the inhibitor concentration, it does suggest that the transient time *is* affected (eq 19), to an extent largely determined by the term $(1 + [Inh]/K_I)$, but additionally influenced by p_c*. Since the K_I of TMP was determined to be 0.19 μM (*2*), the addition of 20 μM TMP would be expected to increase the transient time for the coupled reaction by at least a factor of $(1 + 20/0.19)$, i.e. a factor of 106, relative to its value in the absence of TMP. If no account was taken of this change in transient time, it is possible that the extended lag phase (i.e. long transient time) that would have been observed at 20 μM TMP could have been mistaken for a very low steady state rate. To demonstrate that this is feasible, we need to be able to simulate the time evolution of the coupled enzyme system. We cannot do this using our analytical expressions, since they were obtained by assuming steady state conditions; instead, we have to rely on numerical solution of the appropriate differential equations using a computer, details of which are given under Methods.

Figure 4 shows the expected time course for the free monofunctional enzymes in the presence of increasing amounts of TMP; Figure 5 gives the same plots for the bifunctional enzyme assuming from before that $p_c^* p_r = 0.78$ and that p_c* is independent of inhibitor concentration. In each calculation, the concentrations and kinetic parameters of the two enzymes were identical to those reported by Trujillo et al. (*2*). In the case of the monofunctional enzymes the increase in transient time as the TMP concentration rises is readily apparent, as is the fact that the rates at longer times tend to converge, a result which is in line with our analytical result that the steady state rate should be unchanged by addition of TMP. It should be stressed that this result for the free enzymes is completely free of any uncertainty relating to the concentration dependence of p_c*, since for this case it is always zero: any concern that the conflict between theoretical and experimental results is due to p_c* can therefore be dismissed. In the case of the bifunctional enzyme, similar effects are obtained, but the spread in transient times is far less marked. If we fit straight lines to

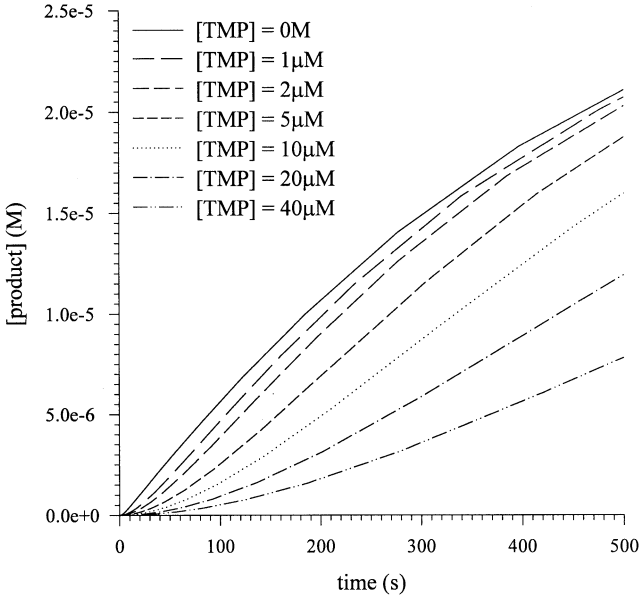

FIGURE 4: Simulated product concentration as a function of time for system of free DHFR and TS enzymes, at various concentrations of the competitive inhibitor TMP.

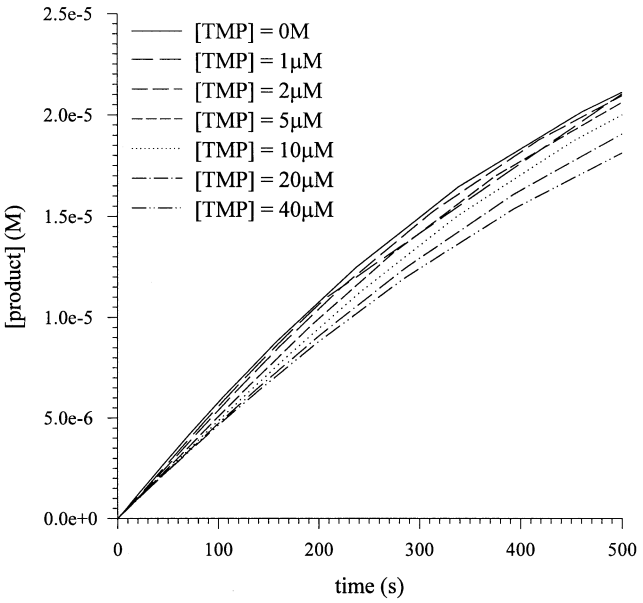

FIGURE 5: Simulated product concentration as a function of time for bifunctional DHFR−TS, at various concentrations of the competitive inhibitor TMP.

each of the curves up to 50 s, we obtain the "apparent steady state" rates plotted in Figure 6, which are qualitatively similar (though by no means identical) to those obtained by Trujillo et al. (*2*). It is worth pointing out that since we obtain the same qualitative behavior whether we use an analytical or a numerical approach, it is clear that the difference between our analysis and the experimental results does *not* result from any of the assumptions made in our theoretical analysis.

CS−MDH: Transient Time Measurements. Kinetics experiments on the CS−MDH fusion protein and its monofunctional analogues were reported by Lindbladh et al. (*5*). As in the case of DHFR−TS, good agreement was obtained between calculated and observed transient times by assuming pseudo-first-order kinetics for the free monofunctional enzymes of the coupled system. As before, then, we apply the pseudo-first-order expressions derived under Methods to

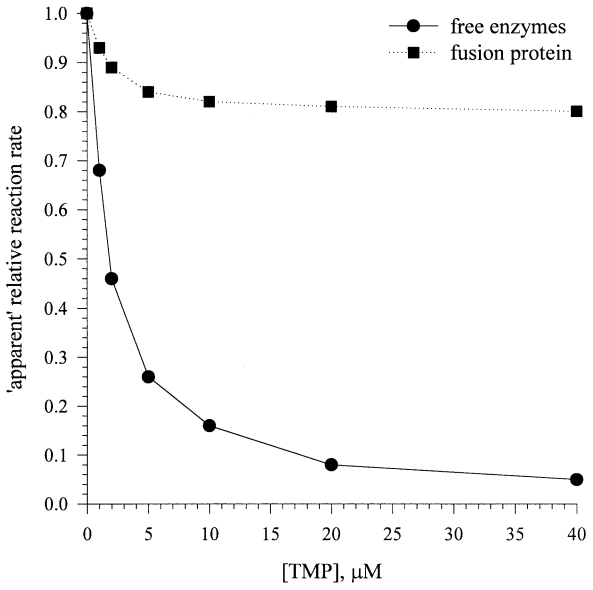

FIGURE 6: Apparent steady state rates obtained from first 50 s of Figures 4 and 5 plotted as a function of TMP concentration.

the bifunctional fusion protein. Under their reaction conditions the expected value of the transient time for the bifunctional enzyme was $\tau = 16$ s. The experimentally observed value was $\tau = 4.4$ s. This means that $1 - p_c p_r = 4.4/16$; therefore, $p_c p_r = 0.73$, a value close to that obtained above for DHFR−TS. In our BD simulations of the same system (*14*), we obtained a transfer efficiency $p_c = 0.45$, a value that is clearly somewhat low compared to the experimental value. Possible reasons for this discrepancy are outlined under Discussion.

CS−MDH: Effects of Adding a Competing Enzyme. The experiments of Lindbladh et al. (*5*) showed that addition of increasing concentrations of AAT caused a dramatic decrease in the activity of the coupled reaction for the free monofunctional enzymes. When the same experiments were carried out with the bifunctional fusion protein, the activity was again decreased, but to a lesser extent: with 10 units of AAT added, the relative activity of the bifunctional enzyme was reduced by ∼30%, while that for the monofunctional enzyme system was reduced by ∼70%. As we now show, these experiments allow us to obtain a second independent estimate of $p_c p_r$. Equation 14 gives us an expression for the expected overall steady state rate of a channeling bifunctional enzyme in the presence of a competing enzyme. To apply this to the data given by Lindbladh et al. (*5*), we need to know both $K_{m,3}$ and $V_{max,3}$, i.e. the kinetic parameters of the competing enzyme AAT. These we obtain by fitting the same expression (eq 14), with $p_c p_r = 0$) to the data given for the free monofunctional enzymes [Figure 4B of Lindbladh et al. (*5*)], using the appropriate values for $K_{m,2}$, $V_{max,2}$, and v_0 given in Lindbladh et al. (*5*). A wide range of $K_{m,3}$ and $V_{max,3}$ values can be used to fit the data equally well; we settled on values of $K_{m,3} = 5 \times 10^{-6}$ M and $V_{max,3} = 1.843 \times 10^{-7}$ M^{-1} s^{-1}, but the results discussed below are completely insensitive to the exact combination of values used. Our value for $K_{m,3}$ is in any case close to the value of 1×10^{-5} M reported by Goldberg and Kirsch (*26*).

Given these fitted values of $K_{m,3}$ and $V_{max,3}$, along with the values of $K_{m,2}$, $V_{max,2}$, and v_0 appropriate to the bifunctional fusion protein, eq 14 was fitted to the experimental data treating the value of $p_c p_r$ as an adjustable parameter

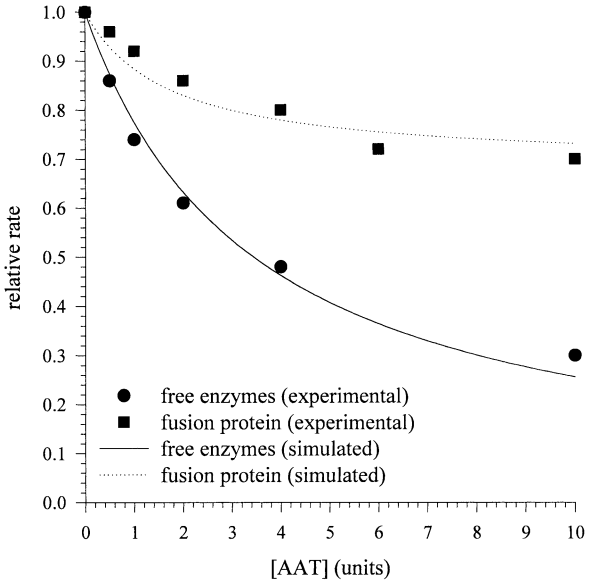

FIGURE 7: Experimentally observed and simulated effects of the competing enzyme AAT on the rate of the coupled CS−MDH system for free enzymes and fusion protein. Experimental data points are estimated from Figure 4B of Lindbladh et al. (*5*).

with the program SigmaPlot (*27*). The best-fit value obtained from this analysis is $p_c p_r = 0.69$, a result that is in excellent agreement with the value ($p_c p_r = 0.73$) obtained from the transient time measurements. Figure 7 compares the best-fit curves obtained with our eq 14 with the experimental data; there is good agreement throughout the range of AAT concentrations studied.

Although the above results fit nicely with the experimental data, there is one apparent discrepancy. Lindbladh et al. report that the transient time for the coupled reaction is *unaffected* by addition of competitor enzyme in both the free enzyme and fusion protein systems. This is inconsistent with our theoretical analysis, since we predict that the transient time should actually decrease as the competitor enzyme concentration increases (eq 13); in the case of the free enzymes, our predicted transient time decreases from 7.9 to 2 s as the AAT concentration is increased from 0 to 10 units. It is not at all obvious why such a discrepancy should exist.

DISCUSSION

In this work we have outlined extensions to the original analyses of Easterby (*19*, *20*) and Ovádi et al. (*21*) that allow us to compare substrate channeling probabilities obtained from simulation directly with experimentally observable steady state rates and transient times. In particular, we have extended the theoretical approach to include the effects of added competitive inhibitors and competing enzymes, both of which have been studied experimentally. Application of our derived expressions to the experimental data obtained for DHFR−TS indicates, as originally stated by Trujillo et al. (*2*), that the substrate transfer efficiency is *at least* 80% under the experimental conditions used. This is in close correspondence with the results of our BD simulations including electrostatic interactions (*13*), in which we found a transfer efficiency of ∼95% at zero ionic strength. Perhaps more importantly, the experimental transfer efficiency is much too high to be consistent with the simulation results obtained in the absence of electrostatic interactions (transfer efficiency ∼5%; *13*). We can state unequivocally then that simple random diffusion is *not* consistent with the efficient substrate channeling observed experimentally, whereas an electrostatically based channeling mechanism is. This point is further emphasized by the preliminary comparison that we report here of experimental and simulation results for nonzero ionic strength (i.e. 0.5 M NaCl), showing that in both cases the transfer efficiency (channeling probability) decreases by a factor of ∼3; an unguided random diffusion mechanism would be expected to be unaffected by changes in ionic strength. It should be noted, however, that to make this comparison more complete, it will be necessary to measure experimentally the K_m of dihydrofolate for DHFR at 0.5 M NaCl. This will allow us to separate out the effects of increasing ionic strength on the kinetic parameters of the enzymes from those on the channeling process itself.

In the case of the CS−MDH system, the experiments reported by Lindbladh et al. (*5*), measuring first transient times and second, the effects of a competing enzyme, allow us to obtain separate and independent estimates of the channeling efficiency. From the transient time measurements a transfer efficiency of 73% is obtained, while from the competition experiments we calculate an efficiency of 69%. Clearly, the two estimates are in excellent agreement. In our BD simulations of the same system (*14*), we obtained a maximum transfer efficiency of 45%, which is somewhat lower than the two experimental estimates. In our original study we noted several reasons why differences might arise, and it is worth briefly restating these points. First, the experiments were performed on yeast enzymes, whereas our simulations used crystal structures of the pig enzymes (no structural data were available for the former): there is 50−60% sequence identity between the yeast and pig forms. Second, our structure of the fusion protein was only a model, based on visual docking of the C termini of CS to the N termini of MDH. The gross structural features of the real fusion protein are likely to be reproduced by such a model, but more subtle features, such as conformational rearrangements in either or both enzymes, are not; such structural changes could conceivably affect channeling efficiencies. In light of the above points, we consider it encouraging that our simulated transfer efficiency approaches the experimentally derived value; it should in any case be noted that as in the case of the DHFR−TS system, our simulated transfer efficiency in the absence of electrostatic interactions (<1%) is totally inconsistent with the experimental results, a point that again underscores the apparent importance of electrostatically based channeling.

Although generally very good agreement is achieved between experimental and simulation results, we have pointed out two areas where qualitatively different results are obtained; these deserve further comment. For the case of added competitive inhibitor we have outlined one possible reason for the discrepancy: our theoretical analysis suggests that the transient time should be drastically increased by the presence of inhibitor, even though the overall steady state rate remains unchanged. Depending on the time scale over which the kinetics experiments are conducted, it is conceivable that a very long transient time *could* be mistaken for a very low steady state rate. The discrepancy obtained in the case of the competing enzyme is, however, more puzzling, since it seems unlikely that the transient time changes predicted by our analysis would be unobservable experimentally. We predict that the transient time decreases from

7.9 to 2 s for the free enzymes when the AAT concentration is raised to 10 units: since Lindbladh et al. (*5*) report transient times down to 4.4 s, our predicted changes should be within the resolution of their experimental equipment. Of course, one could interpret this as implying that our theoretical analysis is simply wrong; however, we have shown that the same approach can be used to obtain separate and independent estimates of the transfer efficiency that are in excellent agreement with one another. Since this latter result lends support to the approach we have adopted, further investigation of the discrepancies outlined here would seem to be warranted.

In examining our theoretical approach, it is clear that we have placed emphasis on use of the product $p_c p_r$ as a measure of substrate channeling efficiency, where before this has been treated as a single variable α (*21*, *22*). This separation of the channeling process into its physical component (diffusion, given by p_c) and its chemical component (reaction, given by p_r) is necessary because our BD simulations address only the former aspect. The partitioning is also of instructive value, however, since it makes it clear that for a channeling mechanism to be of any real advantage, it is *not sufficient* for the enzyme simply to promote a high value of p_c: it is equally important that the kinetic parameters of the second enzyme be such that once a Michaelis complex is formed, there is a high probability that it will then proceed to form product, rather than dissociating. This may seem self-evident, but it is important to state, since it is expected to have consequences for attempts to "engineer" substrate channeling into systems that do not otherwise exhibit channeling.

In conclusion, we have established a direct link between simulation and experimental observables that allows us to assess the role of electrostatics in promoting efficient substrate channeling. We have obtained good agreement between the experimental estimates of transfer efficiencies and the results of computer simulations in which electrostatic interactions are included. Since the simulation results obtained in the absence of electrostatic interactions (i.e. when the substrate is only subject to random diffusion) are much too low ($p_c < 0.05$) to be consistent with the experimental estimates, the results reported here provide further strong support for the idea that efficient substrate channeling in the DHFR−TS and CS−MDH systems is promoted by an electrostatic mechanism.

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