Atomistic Simulations of Competition between Substrates Binding to an Enzyme

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ABSTRACT Although the idea that electrostatic potentials generated by enzymes can guide substrates to active sites is well established, it is not always appreciated that the same potentials can also promote the binding of molecules other than the intended substrate, with the result that such enzymes might be sensitive to the presence of competing molecules. To provide a novel means of studying such "electrostatic competition" effects, computer simulation methodology has been developed to allow the diffusion and association of many solute molecules around a single enzyme to be simulated. To demonstrate the power of the methodology, simulations have been conducted on an artificial fusion protein of citrate synthase (CS) and malate dehydrogenase (MDH) to assess the chances of oxaloacetate being channeled between the MDH and CS active sites. The simulations demonstrate that the probability of channeling is strongly dependent on the concentration of the initial substrate (malate) in the solution. In fact, the high concentrations of malate used in experiments appear high enough to abolish any channeling of oxaloacetate. The simulations provide a resolution of a serious discrepancy between previous simulations and experiments and raise important questions relating to the observability of electrostatically mediated substrate channeling in vitro and in vivo.

INTRODUCTION

A number of enzymes are known to exert electrostatic forces on substrates that promote their productive encounter with the active site (Blacklow et al., 1988; Getzoff et al., 1992; Radic et al., 1997). The relatively long-range nature of electrostatic interactions means that they can operate at distances beyond those at which the atomic details of the molecules are important. As a result, the same attractive forces that act on an intended substrate are equally capable of operating on "incorrect" molecules that happen to share the same essential charge features of the real substrate. As the concentration of these other molecules is increased, it becomes increasingly likely that the substrate will encounter "electrostatic competition" during the process of binding to the enzyme. If the concentration is high enough, electrostatically driven association might be completely suppressed, with the result that the substrate associates at a rate more consistent with a random collision with the enzyme. A familiar manifestation of such a phenomenon is the sensitivity of enzyme–substrate and protein–protein association kinetics to ionic strength. In these cases, the substrate is usually in competition with small inorganic ions such as Na^+ and Cl^- (Radic et al., 1997; Schreiber and Fersht, 1996; Stone et al., 1986). However, a potentially much more important competition effect arises when one considers the potential role played by electrostatic interactions in driving association events in vivo. Many researchers have questioned in conversation, if not in print, whether electrostatic interactions can provide a strong driving force in vivo in the presence of relatively high concentrations of other charged metabolites. That this is a legitimate concern can be illustrated by considering the diffusion-limited enzyme triosephosphate isomerase, a glycolytic enzyme well known to electrostatically accelerate the binding of its substrates/products dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (GAP). Both DHAP and GAP carry charges of ~ -2 e at pH 7 due to their phosphate groups, but this feature is also shared by almost all other substrates in the glycolytic pathway, several of which appear to be present at similar or higher concentrations in the cytosol (Kashiwaya et al., 1994; Srivastava and Bernhard, 1987).

Much of our current understanding of electrostatically driven association processes has come from computer simulations based on Brownian dynamics (BD) methods (Wade, 1996; Gabdoulline and Wade, 2001; Elcock et al., 2001). Up to now, atomically detailed variants of such methods have been limited to simulating the association of two molecules (e.g., proteins), one of which is usually fixed at the center of the simulation coordinate system (Gabdoulline and Wade, 1997). It is to be expected that a similar degree of insight into electrostatic competition effects might also be forthcoming from simulations, but, obviously, this requires the availability of simulation methods capable of handling many molecules at a reasonable level of detail. The present work addresses this issue and reports the application of a many-particle BD simulation method to an explicit biochemical problem for which competition effects are likely to be important. As is discussed, the simulations provide an unprecedented view of the way interactions between a substrate and competing molecules can affect the substrate's association with an enzyme active site. In addition, by providing quantitative estimates of the concentration dependence of competition effects, the simulations

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identify a potential cause of a serious discrepancy between previous simulations and experiments.

The background to the specific problem investigated here is as follows. Several years ago, in their studies of tricarboxylic acid cycle enzymes, Srere and co-workers (Lindbladh et al., 1994) artificially fused citrate synthase (CS) and malate dehydrogenase (MDH) into a single protein in an attempt to circumvent the inherent weakness of the noncovalent interactions known to occur between CS and MDH. Kinetic studies of the malate→oxaloacetate→citrate conversion were performed on the resulting fusion and appeared to indicate that oxaloacetate produced by MDH diffused to the CS active sites without first escaping to bulk solution, i.e., the substrate oxaloacetate was "channeled." Computer simulations using BD techniques supported this conclusion and suggested that channeling was mediated by favorable electrostatic interactions between the negatively charged substrate and a positive electrostatic potential extending over much of the surface of the protein (Elcock and McCammon, 1996; Elcock et al., 1997). Simulations of substrate diffusion indicated that ~45% of oxaloacetate molecules exiting the MDH active site would successfully diffuse to the CS active sites in the absence of added salt, a view that was cemented by further experiments performed by Srere's group (Shatalin et al., 1999). Recently however, Pettersson et al. (2000) have presented more detailed kinetic studies of the same fusion protein that call for a reexamination of these conclusions. Before Pettersson's work, kinetic analyses of CS–MDH were performed under the implicit assumption that the production of oxaloacetate by MDH is an irreversible process. However, the reaction catalyzed by MDH (in the oxaloacetate direction) is highly thermodynamically unfavorable. Pettersson's work has shown that, when account is taken of the reaction's reversibility (together with the potential inhibitory effects of oxaloacetate on the forward reaction), the experimental results are not consistent with channeling of oxaloacetate.

This leaves an open question. How is the experimental observation (or interpretation) of no oxaloacetate channeling to be reconciled with the result from simulations that channeling can occur with an efficiency of up to 45%? One potential explanation of course is simply that the simulations are wrong. This is certainly possible, in part because several assumptions are involved in the simulations, not least of which is the modeling of the fusion protein's structure from structures of the separate enzymes. But aside from these structural uncertainties, there are few other reasons to question the simulations. In addition to performing well in simulating substrate channeling in another bifunctional enzyme, dihydrofolate reductase–thymidylate synthase (DHFR-TS) (Elcock et al., 1996, 1997), BD techniques have been used repeatedly in the past to simulate enzyme–substrate (Wade, 1996) and protein–protein association events (Gabdoulline and Wade, 2001; Elcock et al., 2001) with great success.

An alternative explanation that emerges here is that the discrepancy arises because previous simulations and experiments were, in effect, performed under different conditions. Specifically, the experiments were performed with a 10-mM concentration of malate to ensure that the MDH-catalyzed forward reaction occurs at an appreciable rate. In line with the capabilities of previous programs, the simulations considered only the diffusion of a single oxaloacetate molecule, ignoring the presence of other solutes. But, from the opening to this Introduction, we know that, because malate is structurally almost identical to oxaloacetate, it is as likely to be affected by long-range electrostatic interactions with the enzyme as oxaloacetate. The hypothesis explored here, therefore, is that the high concentrations of malate used in the experiments might be so high that they compete with, and therefore suppress, any channeling of oxaloacetate that might otherwise occur.

To investigate this hypothesis and provide a direct link between simulation and experiment, we report here the extension of previous BD methods to allow simulation of the diffusion of many molecules at once (up to ~300 in the present case). With the new method, BD simulations are performed to obtain the probability of oxaloacetate being successfully transferred to the CS active sites in the presence of varying concentrations of malate. As it turns out, we do indeed find that malate suppresses the channeling of oxaloacetate, and additional simulations demonstrate that this results more because of unfavorable electrostatic interactions between the two types of solute than because of any direct competition between the two for binding to the CS active sites. In addition to providing a novel view of electrostatic competition effects and a potential reconciliation of the previous simulations and experiments, the new results raise serious questions both about how to investigate electrostatically mediated substrate channeling in vitro and its potential importance in vivo.

METHODS

Structures

The modeled structure of the CS–MDH fusion protein used here is essentially identical to that used previously. The two N-termini of the MDH dimer are docked at a short distance from the C-termini of the CS dimer in a way consistent with the presence in the fusion protein of a 3-amino acid linker (Lindbladh et al., 1994). A minor modification in the present work is the use of a NADH-bound structure of MDH in place of the ligand-free structure that was used previously. It is known from kinetics studies that MDH follows an ordered bi-bi- kinetic scheme in which oxaloacetate is released before NADH (Raval and Wolfe, 1962). Because we are interested here in the behavior of oxaloacetate as it leaves the MDH active site, an NADH-bound form of MDH is a more appropriate structure to use. No NADH-bound form of pig mitochondrial MDH is directly available, so a structure was homology-modeled (Sanchez and Sali, 1997) using the NADH-bound *Escherichia coli* MDH enzyme (1emd) as a template with the SWISS-MODEL program (Peitsch, 1996).

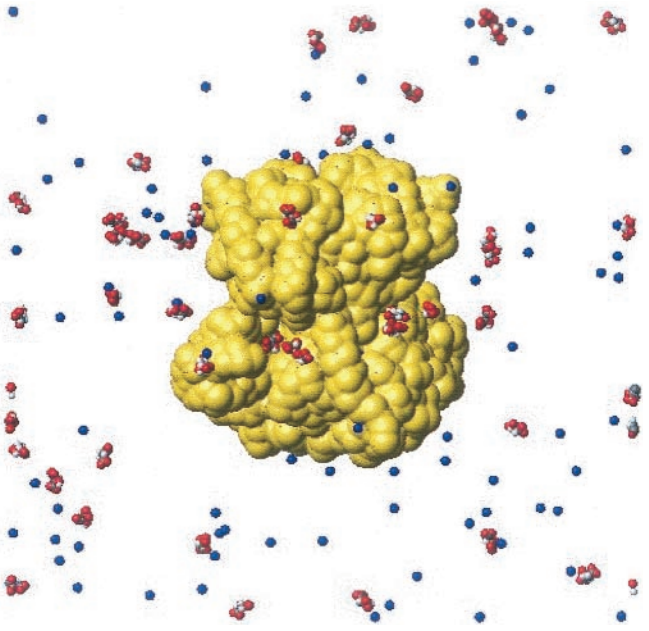

FIGURE 1 Modeled structure of the CS–MDH fusion protein (*yellow*) surrounded by 48 malate molecules (*red*) and 96 sodium ions (*blue*) at the end of a 1-μs period of equilibration (see text for details). The numbers of solute molecules are consistent with that of a 10-mM $(Na)_2$malate solution. This figure was prepared using MolMol (Koradi et al., 1996).

Simulations

All BD simulations were performed with a heavily modified version of the program SDA, originally developed by Gabdoulline and Wade (1997) for the simulation of protein–protein association events. Because the original program is limited to the simulation of two molecules, extensive alterations were required to allow the simulation of multiple molecules. Conceptually, however, the principles remain the same. The motion of each molecule is simulated with the BD algorithm of Ermak and McCammon (1978). Diffusion coefficients for malates and oxaloacetate were set to 0.1 $Å^2ps^{-1}$, those for sodium and chloride were set to 0.2 $Å^2ps^{-1}$. Forces on each molecule are assumed to be solely electrostatic in origin, and stem from the interaction of the effective charges of the molecule with the electrostatic potentials generated by other molecules (Gabdoulline and Wade, 1996). Steric clashes between molecules are prevented by rejecting any simulation step that causes surface atoms to overlap. In the present case, simulations are performed in a periodic cube, at the center of which lies the CS–MDH protein (Fig. 1). The dimensions of this cube (199.76 Å in each direction) are set so that relatively high concentrations of malate can be simulated without the need for prohibitive numbers of molecules. Using this box size, a simulation of a 10-M Na_2(malate) solution, for example, requires the presence of 48 malates and 96 sodium ions. Simulation of a 30-mM NaCl solution, in contrast, requires 144 sodium and 144 chloride ions. In all simulations, periodic boundary conditions are applied so that, as a molecule leaves the box, it returns on the opposite side. Interactions between molecules are determined under the minimum-image convention, i.e., interactions between two molecules occur only through their nearest copies (not through other periodic images).

Electrostatic potentials around each molecule were calculated by solving the Poisson–Boltzmann equation using the finite difference program UHBD (Madura et al., 1995). Charges and radii for all electrostatics calculations were assigned from the CHARMM23 parameter set (MacKerell et al., 1998). Aspartate, glutamate, arginine, and lysine residues were assumed to be in their ionized forms, histidines were assumed to be neutral to be consistent with the experiments for which a pH of 8.1 was maintained. Parameters for NADH, oxaloacetate, and malate were derived by analogy to functional groups in the parameter set. Protein and solvent dielectrics were set to 4.0 and 78.4, respectively. For all solutes, the PB equation was solved on a grid of dimensions 150 $\times$ 150 $\times$ 150. For CS–MDH, the spacing between grid points was 1.5 Å to allow a proper description of its electrostatic potential at long distances. For other solutes, the spacing was 0.5 Å. Except where noted, the ionic strength in all PB calculations was set to zero. The potentials are therefore calculated under the assumption that the solvent is pure water, and that the screening effects due to dissolved ions can be modeled accurately by representing the ions explicitly. For use in the BD simulations, effective charges were assigned to all moving molecules using previously described methods (Gabdoulline and Wade, 1996). For oxaloacetate and malate molecules, a total of four effective charges per molecule were used, placed at the positions of the four carboxylate oxygen atoms.

The starting position of the oxaloacetate was assigned by docking it adjacent to arginine 152 and therefore close to the MDH active site. This location was chosen on the basis of a visual assessment of the possible exit routes for oxaloacetate from its bound position. Initial positions of the malate molecules and sodium ions were assigned by the following procedure. Starting with only the positions of CS–MDH and oxaloacetate determined, 5000 possible positions of a malate molecule were generated at random, and the position with the most favorable electrostatic interaction energy was selected. Then, with the positions of CS–MDH, oxaloacetate, and the first malate molecule assigned, a sodium ion was added after generating 5000 possible positions. After a second sodium ion was inserted, a second malate molecule was added. This process was repeated until the requisite number of molecules was added.

Before simulation of the diffusion of oxaloacetate, the malate molecules and sodium ions were allowed to diffuse freely for a period of 1 μs with the oxaloacetate fixed at its starting position. This long period of equilibration was performed to ensure that the configuration of the system at the beginning of channeling simulations is representative and is not unduly influenced by the somewhat arbitrary way in which coordinates for the malates and sodiums were initially assigned. To assess the likelihood of malate molecules entering the CS active sites during the course of the equilibration simulation, the distance between the C3 atoms of the malates and the CD atoms of the two His-238 residues in CS were continually monitored. Binding of the malate to a CS active site was assumed to occur when this distance dropped below 12 Å.

The entire process of adding malates and sodiums and simulating for 1 μs was conducted ten different times to generate ten different representative states of the system. Channeling simulations were performed starting from each such state, with the diffusion of the malates, sodiums, and now also the oxaloacetate being simulated. The position of the oxaloacetate was monitored throughout. If the C3 atom of oxaloacetate came within 12 Å of CD of one of the two His-238 residues (located in the CS active sites), it was assumed to have successfully channeled. To do this, the oxaloacetate must traverse a distance of ~45 Å, in the course of which journey it may or may not encounter malate molecules (Fig. 2). In contrast, if the C3 atom of oxaloacetate left the confines of the central simulation box, it was assumed to have escaped to bulk solution. When either channeling or escape occurred, the simulation was terminated and a new one started from the original state. This process was conducted 100 times for each of the ten starting states, leading to 1000 different simulations of oxaloacetate diffusion from the MDH active site.

RESULTS

Equilibration

Figure 3 plots the history of each of the malate molecules during the 1-μs period of equilibration of malate and sodium ions in a typical simulation with 10 mM $(Na)_2$malate.

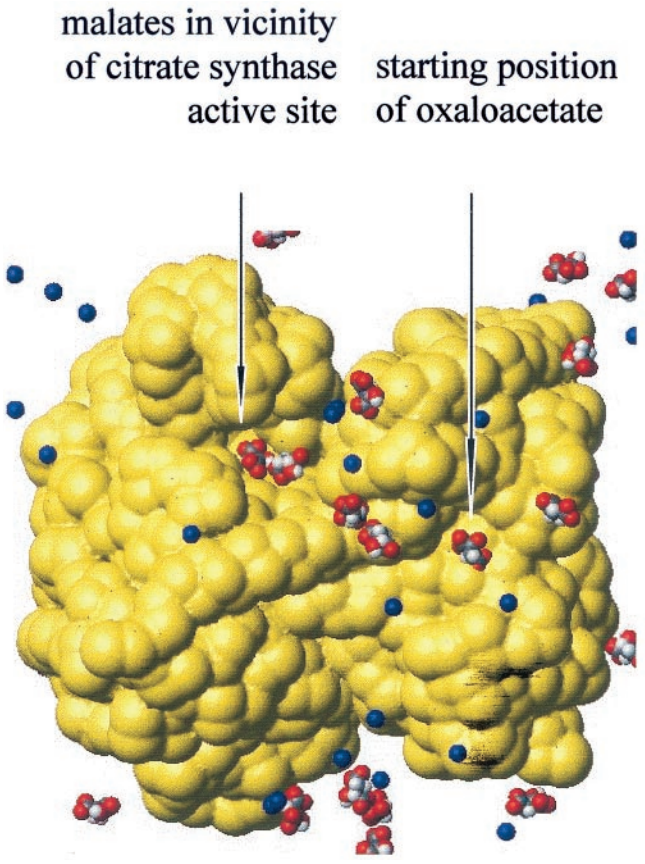

FIGURE 2 Close-up view of the fusion protein, marking the starting position of the oxaloacetate molecule at the entrance/exit of one of the MDH active sites. Notice the string of malate molecules lying between the oxaloacetate and its intended destination, one of the CS active sites.

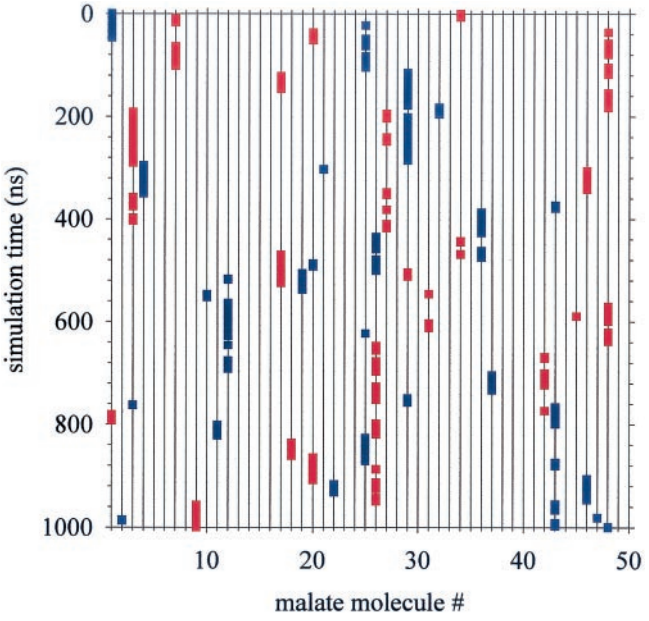

FIGURE 3 History of malate molecules during the 1-μs equilibration period of a 10-mM $(Na)_2$malate solution. Each vertical line plots the history of a different malate. Red and blue squares indicate times at which the malate is occupying the first and second CS active sites, respectively. Note that the size of the square symbols exaggerates the apparent length of time in which malates occupy the active sites.

The figure records for each malate the periods of time at which it occupied either of the CS active sites (see Methods). In the simulation shown, the first CS active site is occupied at some point by 16 of the 48 malates, whereas the second active site is occupied by 21 malates. Interestingly, the two sets are not mutually exclusive. Seven of the malates occupy both active sites at some point during the 1-μs simulation. The longest near-continuous occupation of an active site by a single malate molecule is ~100 ns, although one malate (arbitrarily numbered #26 in Fig. 3) binds on and off for ~300 ns. In total, the first and second CS active sites are occupied 30% and 28% of the time, respectively. The good correspondence between results for the two active sites is a signal that the 1-μs duration of equilibration is sufficient to obtain a representative sampling of malate diffusion events in and around the enzyme. Not surprisingly, given the large distance between the two active sites and the fact that the enzyme is assumed to be rigid in the simulations, events at the two active sites are independent. Both active sites are simultaneously occupied only 8.7% of the time, which is exactly the same as the product 30% $\times$ 28%. In typical simulations with 2.5 and 5 mM $(Na)_2$malate, the active sites are occupied ~18% and 25% of the time, respectively. These numbers allow us to estimate K_c, the concentration component of the equilibrium constant for binding, using the relation,

$$K_c = \frac{[E\cdot M]}{[E][M]},$$

where E, M, and E·M represent the enzyme, malate, and the enzyme–malate complex, respectively, and the square brackets denote their concentrations. For example, the 30% occupancy of the active site with 10 mM $(Na)_2$malate means that the relative proportions of [E·M] to [E] are 30:70. This translates into a value for K_c of 43 M^{-1}. Using the same equation, the calculated equilibrium constants for enzyme–malate binding with $(Na)_2$malate concentrations of 2.5 and 5 mM are 90 and 67 M^{-1}, respectively. The fact that the calculated values of K_c change as a function of malate concentration indicates that there must be a change in activity coefficient of one or more of the binding species. Because any change in the activity coefficient of E is likely to be matched by a similar change in that of E·M, the most obvious culprit is M, malate. In fact, the results indicate that the activity coefficient of malate decreases by a factor of 2 as the concentration increases from 2.5 to 10 mM. It is worth noting that this behavior, although that expected of an ionic solute (Bockris and Reddy, 1970), would be next to

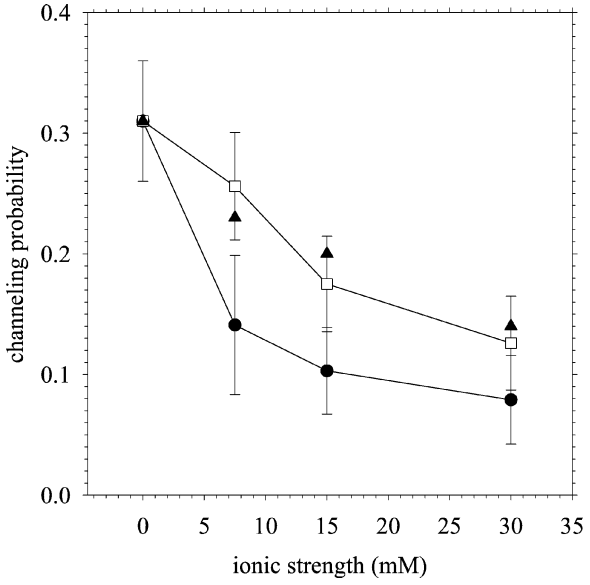

FIGURE 4 Probability of oxaloacetate successfully channeling from MDH to either of the CS active sites as a function of the ionic strength of solutions of $(Na)_2$malate (*circles*), and NaCl (*squares*). Comparison is also provided with the results obtained when the effects of the NaCl solution are modeled implicitly using the Poisson–Boltzmann equation (*triangles*).

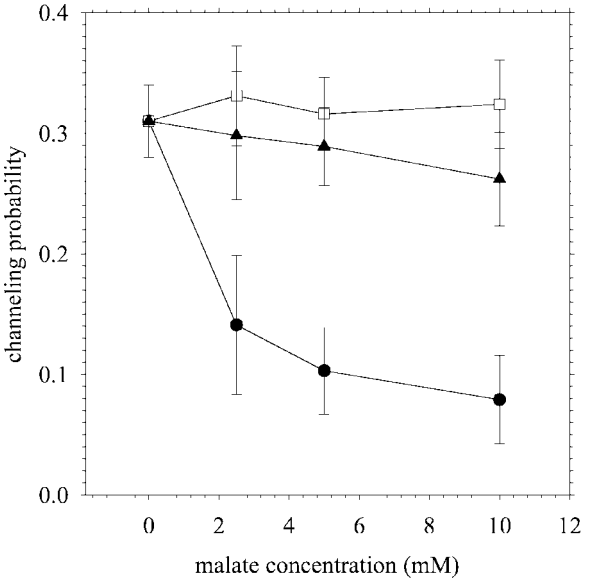

FIGURE 5 Probability of oxaloacetate successfully channeling as a function of the concentration of $(Na)_2$malate, using different interaction models. (*circles*) The same results as in Fig. 4 but replotted against $(Na)_2$malate concentration. (*squares*) Results obtained with uncharged malate molecules and sodium ions. (*triangles*) Results obtained with malate and sodium ions that interact electrostatically with CS–MDH, but which interact only sterically with oxaloacetate (see text).

impossible to capture in a theoretical model that does not treat the solute explicitly.

Channeling simulations

Figure 4 shows the probability of successful channeling of an oxaloacetate molecule from the MDH to the CS active sites as a function of the ionic strength of the $(Na)_2$malate solution. In the absence of malate, the probability of channeling is 0.31. This is somewhat lower than the probability of 0.45 obtained in previous work (Elcock and McCammon, 1996), which can probably be attributed to the use here of a slightly different structure of the CS–MDH fusion protein, a more detailed model of the substrate, or a different starting position (see Methods). The more important point however, is that, as the concentration of malate is increased from 0 to 10 mM, the probability of successful channeling decreases by a factor of more than four to ~7% (Fig. 4, *circles*). The suppressing effects of a $(Na)_2$malate solution are compared with those of a NaCl solution of identical ionic strength in the same figure (*squares*). For a given ionic strength, the channeling suppressing effects of malate are clearly greater than those of chloride, even though there are three times as many chloride ions as malates in corresponding simulations. Also shown in Fig. 4 are the results of channeling simulations in which the effects of NaCl are modeled implicitly by recalculating the electrostatic potential for CS–MDH for each ionic strength (*triangles*). In such simulations, only CS–MDH and oxaloacetate appear explicitly. This latter method, which relies on the Poisson–Boltzmann equation to provide an adequate description of ionic strength effects, is the method that has been used in all previous BD simulations of association kinetics. It is encouraging to see, then, that the calculated effects of NaCl on channeling using the explicit and implicit representations are more or less identical.

In principle, the observed suppression of substrate channeling by malate could be caused by two potential factors. One possibility is that the presence of malates, which are clearly attracted to the CS active sites (Fig. 3), sterically prevents oxaloacetate from reaching the active sites. An alternative possibility is that the presence of the negatively charged malates results in a decrease in the positive electrostatic potential in the vicinity of the enzyme, making it less attractive for oxaloacetate to bind. Simulations allow us to assess both possibilities in a way that is not possible to perform experimentally. Figure 5 compares the results first shown in Fig. 4 (*circles*) with those obtained from simulations in which uncharged malate molecules and sodium atoms are used in place of the proper, charged forms (*squares*). In the latter simulations, the malates and sodiums effectively function solely as "filler" that simply excludes volume (albeit not very much). As expected, in these cases, the channeling probability is completely insensitive to the concentration of the malate. The concentrations studied here are far below those at which excluded volume effects might become important (Ellis, 2001). The same figure also shows the results of hybrid simulations in which the malates and

sodiums are charged and interact fully with the CS–MDH fusion protein, but in which their electrostatic interactions with oxaloacetate are ignored. These simulations allow us to determine whether malate can suppress channeling by simply occupying the active site. Including electrostatic interactions between the malates and CS–MDH ensures that the spatial distribution of the malates is realistic, i.e., that they have a good chance of being found in the CS active site. In these simulations, there does appear to be a very slight suppression of channeling (Fig. 5, *triangles*), but because this is nowhere near large enough to account for the effects observed in the full simulations, we can conclude that the primary contribution to channeling suppression comes from unfavorable electrostatic interactions between malate and oxaloacetate.

DISCUSSION

The simulations reported here raise a number of important new points. The first and most general point is that the extension and application of atomically detailed BD methods to meaningful simulation of many-particle systems is now a feasible undertaking. The present example provides a glimpse of the kinds of findings that might be obtained from simulations that take explicit account of interactions between multiple solutes. Of course, the result that malate strongly suppresses substrate channeling is not a hugely surprising result. After all, the simulations were performed in the first place to investigate just this hypothesis. In contrast, the finding that the activity coefficient of malate (reflected in its binding to the CS active sites) drops dramatically as its concentration increases is a completely unanticipated (but, in retrospect, reasonable) result. In addition, the demonstration that the suppression of channeling is due primarily to unfavorable electrostatic interactions between the malate and the oxaloacetate (Fig. 5) indicates the potential power of simulations for providing biochemical insight that would otherwise be difficult to obtain. This is particularly important to note, given that our current understanding of competition effects (such as those implicit in enzyme inhibition) is really only phenomenological. Although they can be described by formulation of suitable chemical rate equations, such descriptions provide no true atomic-level understanding. Computer simulations might ultimately enable such an understanding to be attained.

The second major point to make is that, in its first application to a specific biochemical problem, the new simulation methodology has provided a potential resolution of serious discrepancies between previous simulations and experimental results for the CS–MDH fusion protein. The simulations indicate that the malate concentrations used in the experiments are sufficient to reduce channeling to ~7%, a level sufficiently low that it is unlikely it would be observed at all, and this is despite the fact that the equilibrium constant for malate binding to the CS active sites is actually very low. This point should be further reinforced when it is considered that, in addition to 10 mM malate, the experiments were also conducted with a 40-mM phosphate buffer (Lindbladh et al., 1994; Shatalin et al., 1999). Figure 4 shows that a simple inorganic salt such as NaCl, although not as effective as $(Na)_2$malate, is nevertheless more than capable of diminishing channeling. Because of this, we can see now that electrostatically driven channeling would be unlikely to occur in the experiments, even if (in the absence of other competing molecules) the fusion protein was technically capable of it.

These results, in turn, raise important questions. First, how is substrate channeling to be measured in systems such as CS–MDH? The experimental use of 10 mM malate was originally made (quite reasonably) because the equilibrium of the malate→oxaloacetate conversion lies strongly in favor of the reactants. The high concentration was used under the assumption that it would ensure that the forward reaction was essentially irreversible. Pettersson's work has since shown that this was not correct. The concentration of oxaloacetate soon reaches a level at which the reverse reaction becomes noticeable and important (Pettersson et al., 2000). Even higher concentrations of malate could, of course, be used to avoid this problem, but the present results indicate that this approach would just further abolish any chance of channeling occurring. In fact, to demonstrate channeling in CS–MDH at all would appear to require dropping both the malate concentration and ionic strength of the buffer substantially. This can certainly be done. Numerical analysis of the observed kinetics of coupled enzyme systems does not require that the first enzyme operate irreversibly (although this condition certainly helps in formulating analytical solutions to the rate equations). Having said that, it should be noted that there are other reasons why electrostatic substrate channeling might not ultimately be observed in CS–MDH experimentally. The current simulations are performed under two structural assumptions: first, that the overall structure of the protein can be modeled by docking separate structures of CS and MDH together, and second, that the starting point for oxaloacetate's diffusion can be identified simply on the basis of a purely visual examination of the active site structure of MDH. Neither assumption is likely to affect the most important result reported here: that increasing concentrations of malate progressively disrupts substrate channeling because malate electrostatically competes with the channeling metabolite. However, alternative structural models of the fusion protein may well differ significantly in their basal levels of channeling, i.e., in their ability to efficiently channel oxaloacetate in the absence of malate. Because of this, it is important to add the unfortunate caveat that, even in properly designed experiments, it may eventually be found that the CS–MDH fusion protein does not channel substrate. If this proves to be the case, it need not be viewed as a death knell for the idea of electrostatic substrate channeling (Knighton et al., 1994). The fusion protein is,

after all, an entirely artificial construct, produced in an attempt to circumvent the problem of the low stability of interactions between CS and MDH. It is frustrating to note that the inherent weakness of many enzyme–enzyme interactions remains a fundamental impediment to their experimental study.

The final important question is what these results mean for prospects for electrostatic substrate channeling occurring in vivo. This is a simple question to raise, but, at the moment, a near impossible one to answer. Clearly, the present results indicate that electrostatic channeling of a substrate is likely to be sensitive to competition from other charged metabolites. But to assess just how sensitive it is requires knowing the local concentrations of potential competing metabolites. These can be extremely difficult to obtain for a number of reasons, not least of which is that it is often difficult to assess the relative proportions of free and enzyme-bound forms of the metabolite (Fell, 1997). Recent experimental advances suggest that proper quantitative measures of metabolite concentrations may soon be attainable (Fiehn et al., 2000). Together with accurate measures of the distributions and concentrations of enzymes, it should, in future, be possible to develop a conceptual framework suitable for answering this question.

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