Accelerated Publications

Evidence for Electrostatic Channeling in a Fusion Protein of Malate Dehydrogenase and Citrate Synthase†

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Received June 20, 1996; Revised Manuscript Received August 2, 1996⊗

ABSTRACT: Brownian dynamics simulations were performed to investigate a possible role for electrostatic channeling in transferring substrate between two of the enzymes of the citric acid cycle. The diffusion of oxaloacetate from one of the active sites of malate dehydrogenase (MDH) to the active sites of citrate synthase (CS) was simulated in the presence and absence of electrostatic forces using a modeled structure for a MDH−CS fusion protein. In the absence of electrostatic forces, fewer than 1% of substrate molecules leaving the MDH active site are transferred to CS. When electrostatic forces are present at zero ionic strength however, around 45% of substrate molecules are successfully channeled. As expected for an electrostatic mechanism of transfer, increasing the ionic strength in the simulations reduces the calculated transfer efficiency. Even at 150 mM however, the inclusion of electrostatic forces results in an increase in transfer efficiency of more than 1 order of magnitude. The simulations therefore provide evidence for the involvement of electrostatic channeling in guiding substrate transfer between two of the enzymes of the citric acid cycle. Similar effects may operate between other members of the citric acid metabolon.

The association of enzymes involved in a particular metabolic pathway into a single multienzyme complex is a well-known phenomenon, with perhaps the most prominent example being the pyruvate dehydrogenase complex (Reed, 1974) responsible for production of acetyl coenzyme A. The purported advantages of forming such multienzyme complexes seem intuitively reasonable (Welch, 1977; Srere, 1985): the close proximity of enzymes responsible for catalyzing consecutive steps of a metabolic cycle may reduce the time required for substrate to diffuse between enzymes and prevent the substrate from escaping into solution where it might be sequestered by other enzymes for use in different metabolic pathways. This latter aspect may be particularly important for maintaining metabolic flux with low overall concentrations of metabolite (Welch, 1977).

In principle, consecutive enzymes in a multienzyme complex might be close enough together that simple random diffusion would be sufficient to provide an efficient means of transferring substrate between active sites. Recent experimental and theoretical work suggests however that the efficiency of substrate transfer might be dramatically enhanced by electrostatic effects. This idea stems largely from the solution of the crystal structure of the protozoan bifunctional enzyme dihydrofolate reductase−thymidylate synthase (DHFR−TS)[1] (Knighton *et al*., 1994). DHFR and TS catalyze sequential steps in the thymidylate cycle, with the dihydrofolate (FH_2) produced by the TS-catalyzed reaction subsequently acting as the substrate for the DHFR-catalyzed reaction. Experimentally the bifunctional form of the enzyme has been shown to have significant kinetic advantages over the related monofunctional enzymes in terms of a markedly reduced lag time for appearance of the final products of the coupled system (Meek *et al*., 1984). The crystal structure of DHFR−TS shows the entrances to the active sites to be at the enzyme surface and separated by around 40 Å. Lying between the two active sites is a number of positively charged residues which were hypothesized to guide FH_2, which has a net charge of −2e, from the TS to the DHFR active site. Strong support for this idea has come from Brownian dynamics (BD) simulations of the process of substrate transfer (Elcock *et al*., 1996). In simulations conducted at zero ionic strength the efficiency with which substrate was transferred from the TS to the DHFR active site was found to be almost 100%. Perhaps more importantly, the simulations suggested that uncharged substrates, which experience no electrostatic guidance mechanism, are transferred with an efficiency of only around 5%: much too low to explain the experimental observation of efficient channeling. Further support for electrostatic channeling in DHFR−TS has been provided by recent experimental work showing that the efficiency of substrate transfer is dependent on ionic strength (*Trujillo et al*., 1995, 1996); this result,

† This work was supported by the Wellcome Trust under Grant 041157/Z/94/Z and by grants from the National Institutes of Health, the National Science Foundation, and the National Science Foundation MetaCenter Program.

⊗ Abstract published in *Advance ACS Abstracts,* September 1, 1996.

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

S0006-2960(96)01474-2 CCC: $12.00

which was also reflected in the simulations, is an expected characteristic of an electrostatic mechanism of transfer.

Given the apparent importance of electrostatic effects suggested by both experimental and theoretical results for DHFR−TS, it is clearly of considerable interest to ask whether a similar mechanism operates elsewhere in nature. The focus of the present work is therefore on investigating the possible role of an electrostatic channeling mechanism in transferring substrate oxaloacetate between the citric acid cycle enzymes malate dehydrogenase (MDH) and citrate synthase (CS). Recent experimental work (Lindblah *et al*., 1994) has described the preparation and characterization of a fusion protein of the yeast forms of the two enzymes in which the C-termini of CS are connected by a short linker sequence (Gly-Ser-Gly) to the N-termini of MDH. Kinetics studies of the fusion protein resulted in two findings that suggest that oxaloacetate is efficiently channeled between MDH and CS. First, the lag (transient) time for formation of the final products (NADH and citrate) in the fusion protein was shorter than in the free enzymes; the lag time was also shorter than the theoretical estimate based on the ratio of v_{max} and K_m for the CS part of the coupled system (Easterby, 1973). Second, addition of aspartate aminotransferase (AAT), which competes with CS for the intermediate substrate oxaloacetate, had much less of an effect on the steady-state rate of the fusion protein than that of the free enzymes, an observation that indicates that oxaloacetate is largely sequestered from the bulk solution.

From a simulation viewpoint the MDH−CS fusion protein is attractive since good structural information, a prerequisite for theoretical work, is available for each of the individual components of the system. While crystal structures of the yeast forms of the enzymes have not been solved, structures are available for the pig heart forms of both enzymes, and these are expected to be good models for the yeast enzymes. The problem of docking the two enzymes together in some reasonable fashion remains of course, but the use in the experimental work of a very short linker region (three amino acids) to connect the CS and MDH enzymes means that the orientational possibilities for such a fusion protein are drastically reduced. In fact, this sufficiently constrains the system that it is possible for a model of the fusion protein to be constructed by simply docking the CS and MDH structures so that the CS C-termini are adjacent to the MDH N-termini (Lindblah *et al*., 1994).

A model of the fusion protein built in this way has the MDH and CS active sites separated by nearly 60 Å, with the MDH active site facing away from CS. This is such a large distance that it would appear at first sight unlikely that substrate would be transferred between active sites with any great efficiency: certainly, it appears to have prompted Srere and co-workers to consider the possibility of a dimer of the fusion protein being responsible for efficient channeling (Lindblah *et al*., 1994). We report here, however, the results of BD simulations which suggest that, even in a monomer of the fusion protein, an electrostatically based channeling mechanism can be surprisingly efficient.

MATERIALS AND METHODS

All BD simulations were performed with the program UHBD (Madura *et al*., 1994, 1995). The crystal structures of MDH (Gleason *et al*., 1994) and CS (Remington *et al*., 1982) (both of which are dimers) were used as the starting point for generating a model of the fusion protein. The "open" form of CS was used as it has been suggested to represent a substrate entry/product release form of the enzyme (Remington *et al*., 1982). Bound citrate molecules were removed from both the MDH and CS structures. Hydrogens were added to each enzyme using the molecular simulation program CHARMM (Brooks *et al*., 1983). Titratable residues were assumed to be in their usual protonation state at pH 7; i.e., the net charge of all Asp and Glu residues was set to −1e and all Arg and Lys residues set to +1e; protonation states for histidines were assigned on the basis of their local environment, and all were in neutral forms. The total charge on the MDH dimer was therefore +6e; that on the CS dimer was −2e. The two enzymes were docked so that the C-termini of the CS dimer were within 5 Å of the N-termini of the MDH dimer and so that structural overlap was kept to a minimum (Figure 1). A more refined method of docking involving energy minimization of the resulting structure could of course be envisaged, but the structural uncertainties involved are large enough at this stage that we feel that such an approach would not be justified; in any case, the results are not likely to be altered significantly.

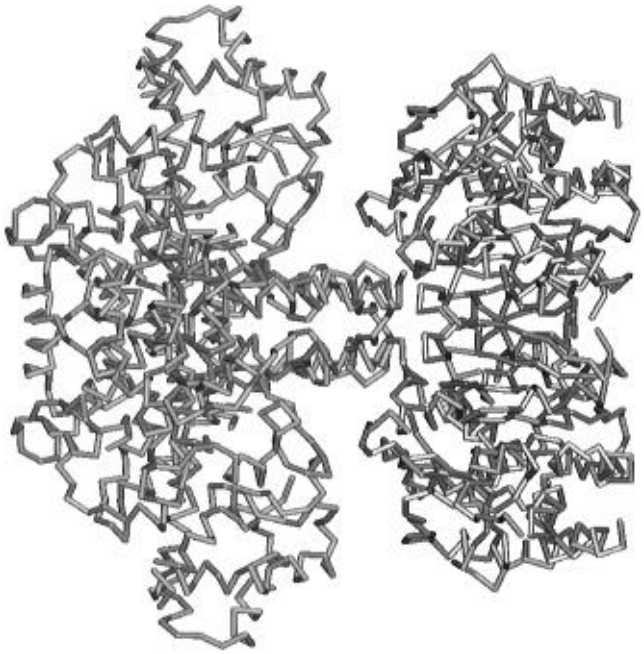

FIGURE 1: Cα trace of the MDH−CS fusion protein model. The CS dimer is shown in red, with the three C-terminal residues of each monomer colored purple. The MDH dimer is shown in blue, with the three N-terminal residues of each monomer colored green. This figure was prepared using the program QUANTA.

Prior to BD simulations being performed, the electrostatic potential around the fusion protein was obtained by finite-difference solution of the linear Poisson−Boltzmann equation (Honig & Nicholls, 1995) using a 160^3 grid of spacing 1.2 Å. Boundary potentials were set assuming that each charge in the system is subject to Debye−Hückel screening. Atomic radii and charges were obtained from the CHARMM22 parameter set (Mackerell *et al*., 1995). The internal dielectric of the protein was set to 4.0 while that of the solvent was set to 78.0, appropriate for water at 25 °C. The ionic strength was set to 0 mM in most simulations, although a range of values between 0 and 150 mM was used to investigate the ionic strength dependence of transfer efficiency. Oxalo-

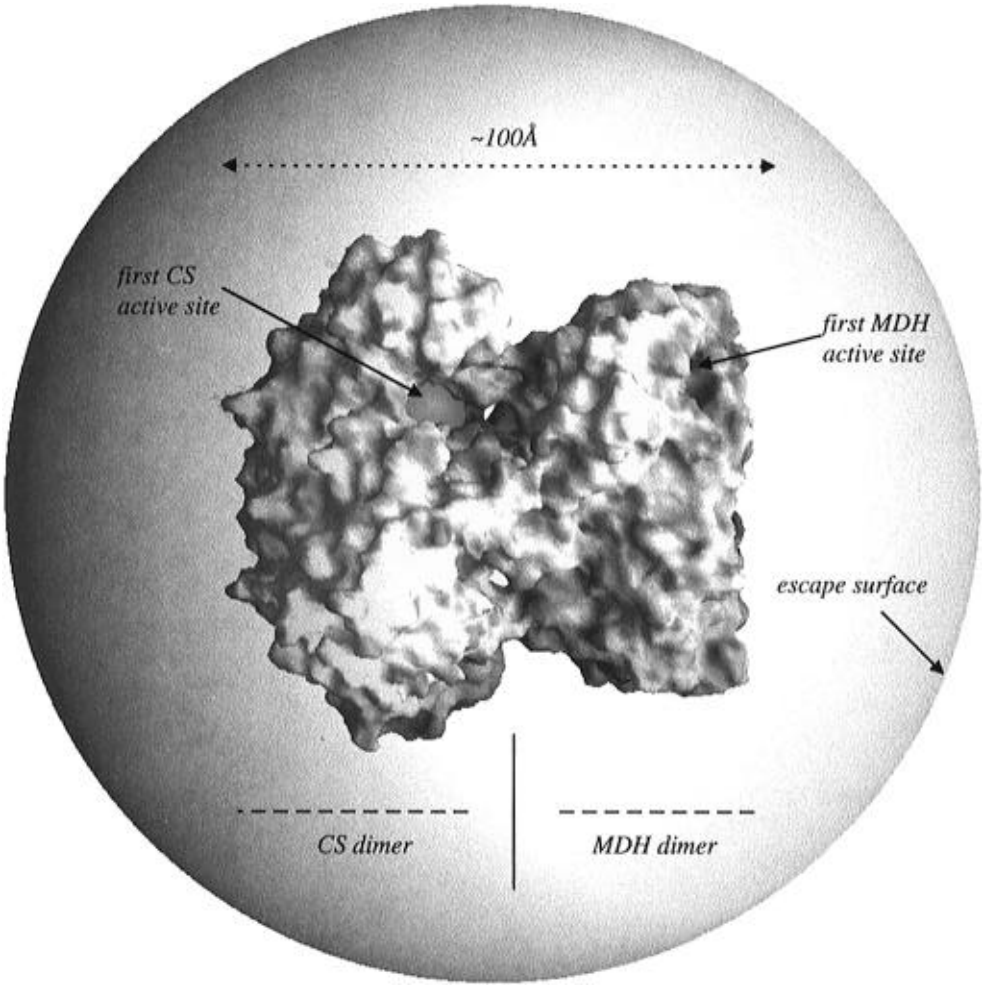

FIGURE 2: MDH−CS system viewed from a similar position to that in Figure 1. The molecular surface of the protein is colored according to the electrostatic potential calculated at zero ionic strength. Blue areas on the molecular surface represent electrostatically positive regions; red, electrostatically negative regions (limits set at ±15 kT). The green area indicates the substrate starting point at the first MDH monomer's active site. The purple area marks the substrate reaction region at the first (most favored) CS active site. The second (less favored active site) CS active site is around the back of the figure. Substrate molecules reaching the purple surface are assumed to react at the CS active sites. The large sphere marks a radius 90 Å from the center of the protein: substrate molecules which reach any point on this surface are considered to have escaped to bulk solution. This figure was prepared using the program GRASP.

acetate was modeled as a sphere of radius 2 Å and charge −2e; as in previous work (Elcock *et al*., 1996) this does not represent a particularly realistic description of the substrate's atomic level structure, but electrostatic channeling effects, *if present*, are likely to be largely accounted for by the overall charge of the substrate. Simple spherical models of substrate have worked well in other simulations, for example in studies of the diffusion of acetylcholine to the active site of acetylcholinesterase (Antosiewicz *et al*., 1995). A series of BD simulations were performed starting from 1180 different positions evenly distributed over a surface 12 Å from the position of the C1 atom of the bound citrate molecule in the active site of the first MDH monomer. These starting points are located adjacent to the flexible loop formed by residues 77−87 known to play a role in substrate binding (Gleason *et al*., 1994); these points therefore appear to be a reasonable model for the location of oxaloacetate emerging from the MDH active site. Substrate trajectories were simulated using the Ermak−McCammon algorithm (Ermak & McCammon, 1978) with a time step of 0.05 ps. Trajectories were propagated until one of the following criteria were satisfied: (1) the substrate "reacted" at the CS active sites; (2) the substrate reached a distance of 90 Å from the center of the fusion protein (at which point it was assumed to have escaped to bulk solution (see Figure 2)); (3) the trajectory exceeded 1×10^6 steps. The last criterion was required because a few simulations remained trapped at the MDH active site where they would have stayed indefinitely; such trajectories were omitted from the analysis of results. A "reaction" was assumed to occur when the substrate came within 8 Å of the position of the C3 atom of the bound citrate molecule observed at the active sites of the CS crystal structure (Remington *et al*., 1982). An 8 Å distance was selected since it gives a reaction region small enough that it provides a good definition of the active site area but sufficiently large that it is unaffected by changing the molecule's grid positioning; the results reported here are not sensitive to this choice of reaction distance. Substrate transfer efficiency was calculated as the percentage of trajectories which terminate successfully at one or other of the CS active sites.

RESULTS

In the simulations, the efficiency of substrate transfer from the MDH active site to either of the CS active sites is strongly dependent on the net charge of the substrate (Figure 3), dropping sharply as the charge is changed from −2e (that of oxaloacetate) to +2e. A more important result is that

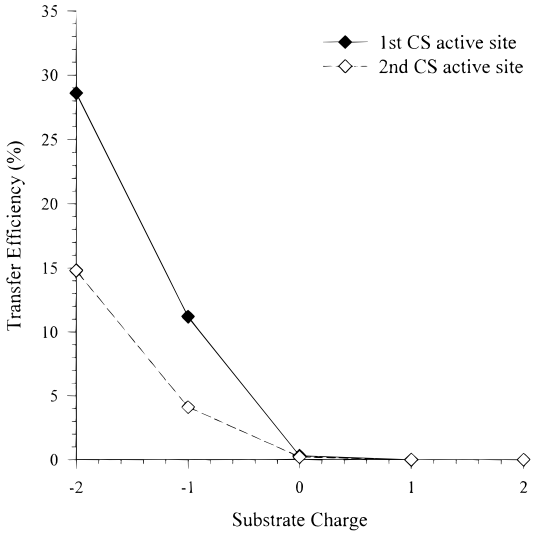

FIGURE 3: Dependence of substrate transfer efficiency on substrate charge at zero ionic strength. Transfer efficiency is defined here as the percentage of substrate molecules leaving the MDH active site which reach a CS active site.

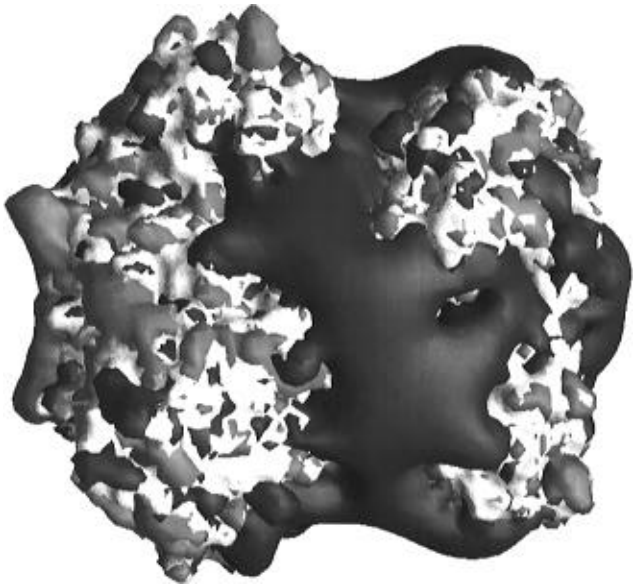

FIGURE 4: Electrostatic isopotential contours around the MDH−CS fusion protein (calculated at zero ionic strength) viewed from the same position as in Figure 2. The blue surface connects all points having a potential of +1.5 kT; the red surface connects all points having a potential of −1.5 kT. Note the presence of a continuous region of positive electrostatic potential covering the area where the MDH and CS dimers meet.

the transfer efficiency for an uncharged substrate, which is not subject to any electrostatic channeling effects (and therefore behaves in exactly the same way as a charged substrate would with the enzyme's electrostatic field switched off), is only around 0.5 ± 0.4%. This is perhaps what one would expect, given the relative positioning of the active sites in the model of the fusion protein (Figure 2). In contrast, the transfer efficiency for a charge of −2e is 43 ± 2%. This result, which does not seem at all obvious from simple inspection of the model structure, represents an approximate 100-fold increase in efficiency over the simulations without electrostatic channeling effects. On the other hand, the result is less surprising when the electrostatic potential around the fusion protein is examined (Figure 4); isopotential contours marked at values of ±1.5 kT indicate the presence of a large area of positive (i.e., favorable) electrostatic potential extending over the region where MDH and CS meet. That the transfer efficiency does not exceed 50% appears to be due to the fact that the region of favorable potential does not quite extend to the substrate starting positions, though the apparently unfavorable positioning of the MDH active site probably also plays a role. Once substrate molecules reach the region of favorable electrostatic potential however, they are likely to remain within it and, once trapped in this way, stand a good chance of ultimately diffusing to one or other of the CS active sites.

Simulations performed to assess the relative contributions of the two enzymes in determining the efficiency of substrate transfer suggest that both play important roles, though CS dominates. When the electrostatic field due only to MDH is switched off (i.e., when all atomic charges on MDH are set to zero), the transfer efficiency is reduced by a factor of 4 to 12 ± 2%; when the electrostatic field due only to CS is switched off, the transfer efficiency is reduced even more dramatically, to only 0.6 ± 0.5%. This apparent dominance of CS's electrostatic field was further emphasized by the results of simulations run in the opposite direction, i.e., starting at the CS active site and terminating at the MDH active sites. In the DHFR−TS system such "backward" simulations were of comparable efficiency to the "forward" results, suggesting that in DHFR−TS the role of electrostatics is more one of restricting the substrate's diffusion to the region between the active sites than one of actively guiding substrate from one active site to the other (Elcock *et al.*, 1996). Results for the MDH−CS system are more difficult to interpret since all backward trajectories remained trapped indefinitely at the CS active site, making it impossible to calculate substrate transfer efficiencies. However, since this trapping results from strong electrostatic effects (no trapping was observed when the enzyme's electrostatic field was switched off), it is tempting, and perhaps not unreasonable, to interpret this result as implying that, in contrast to DHFR−TS, the electrostatic channeling in the MDH−CS system is indeed directional.

Consistent with the above results suggesting the importance of electrostatic effects, the transfer efficiency obtained from the simulations is found to be strongly dependent on the ionic strength of the solution (Figure 5); at 150 mM the efficiency drops to a value of 11 ± 2%. Again, this result is understandable in terms of the electrostatic potential (Figure 6); the increased shielding of the fusion protein's charged residues means that the regions of favorable electrostatic potential no longer extend out so far into solution so that the probability of substrate escape is increased.

In the model that we have constructed substrate molecules start their trajectories at one end of the fusion protein (Figure 2). Since it is possible for the substrate to diffuse from this position around either side of the protein, reactions are expected to be obtained at both of the CS active sites. However, as the substrate starting positions are slightly displaced toward one side of the protein, it is perhaps not surprising that an approximately 2-fold preference is obtained for reaction at one of the CS active sites (from here on, we use "first" active site to mean "more favored") (Figures 3 and 5). More interesting is the fact that the distributions of reaction times (i.e., trajectory lengths) for reactions at the two CS active sites are very different (Figure 7). For the less-favored CS active site, the probability of a particular

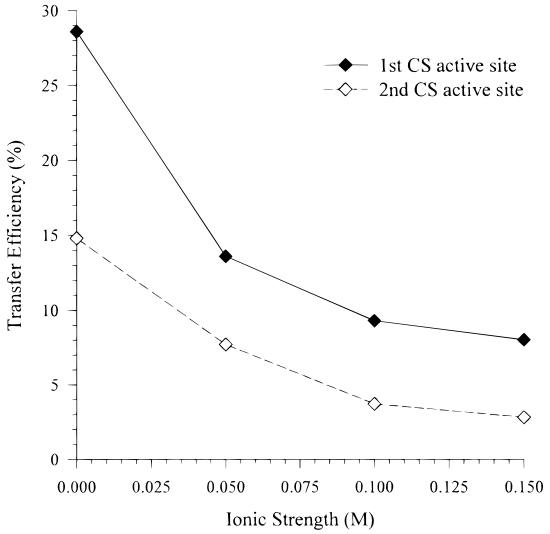

FIGURE 5: Dependence of substrate transfer efficiency on ionic strength for a substrate charge of −2.

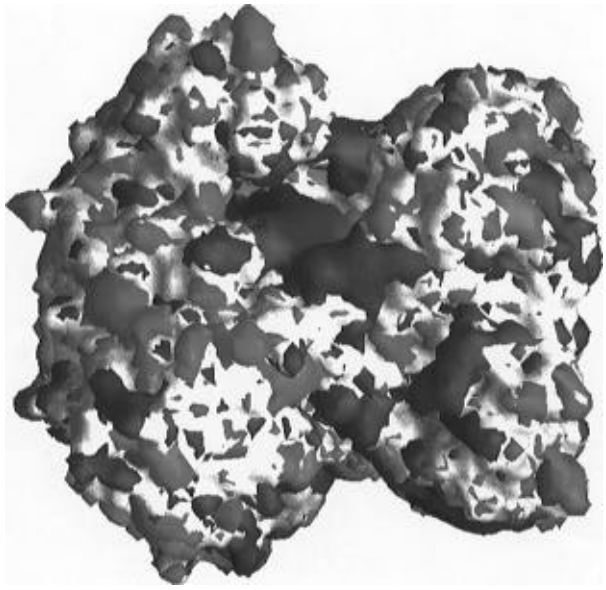

FIGURE 6: Electrostatic isopotential contours around the MDH−CS fusion protein (calculated at 150 mM) viewed from the same position as in Figure 2. The blue surface connects all points having a potential of +1.5 kT; the red surface connects all points having a potential of −1.5 kT.

reaction time increases monotonically as a function of the reaction time itself. In contrast, for the more favored CS active site, a distinct maximum in the distribution is observed at much shorter times, superimposed on a trend similar to that seen with the second CS active site (Figure 7). In other words, a considerable proportion of the reactive trajectories for the first CS active site reacts *very* rapidly: out of 438 trajectories which terminate at the first CS active site, 113 (26%) do so in less than 10 ns, whereas only 4 out of 227 reactive trajectories (2%) at the second CS active site react within this time. The reasons for this dramatic difference are not immediately obvious, but it is interesting to note that no correlation appears between the substrate's starting position and either its eventual fate or its speed of reaction; any concern that the results might be very sensitive to the former aspect can therefore be ruled out. Instead, it appears likely that the bimodal distribution reflects real differences in substrate trajectories, though it is by no means clear of course whether these effects will ultimately be of much

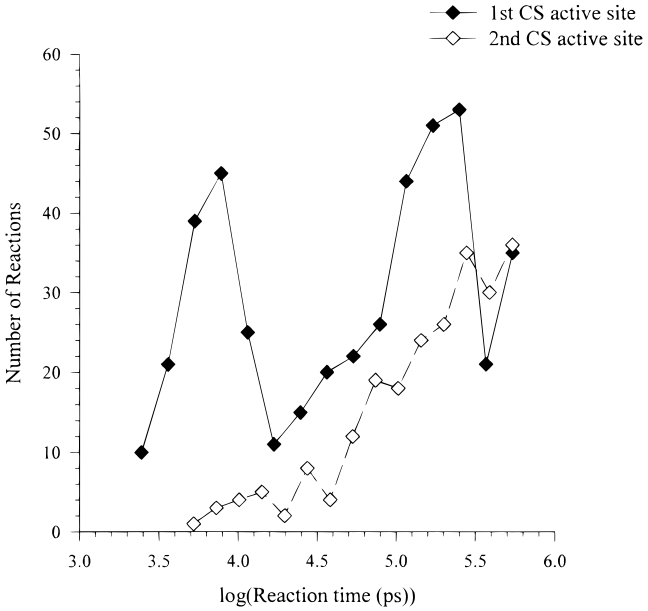

FIGURE 7: Distribution of reaction times (i.e., trajectory lengths) for reaction at the two CS active sites obtained at zero ionic strength.

relevance to overall channeling efficiency; further work will be required to address this problem more closely.

DISCUSSION

The simulation results reported here provide evidence that the experimental observation of efficient substrate channeling in the MDH−CS fusion protein is due to an electrostatic mechanism. Since electrostatic effects appear so important in the simulations, it is of course no surprise to find that if channeling is very efficient for a substrate charge of −2e (as it is here), it is extremely inefficient for a substrate charge of +2e. On the other hand, comparison with results obtained for a substrate charge of zero is more illuminating since it gives not only a measure of the increase in transfer efficiency that might result from electrostatic channeling but also an indication of whether electrostatic channeling is required at all in the first place. In the present case, the MDH and CS active sites are separated by around 60 Å, and in the absence of electrostatic effects (i.e., when the substrate has a charge of zero) much less than 1% of substrate molecules leaving the MDH active site reach the CS active site. The simulations suggest therefore that efficient channeling of substrate does not result simply from the proximity of the CS and MDH active sites. When electrostatic effects are included however (i.e., when the substrate has a charge of −2e), the transfer efficiency not only increases by around 2 orders of magnitude but also approaches a value that is more consistent with the experimental observation of efficient substrate channeling.

Many of the results reported here for the MDH−CS system parallel those we obtained previously in our studies of channeling in DHFR−TS (Elcock *et al.*, 1996) and are obviously those to be expected of an electrostatic channeling mechanism. The ionic strength dependence of transfer efficiency obtained here appears more marked than in our study of the DHFR−TS system, but not surprisingly follows the same qualitative trend: increasing ionic strength tends to suppress any electrostatic channeling effect which might be present. The magnitude of this ionic strength effect is likely to vary from system to system and, in extreme cases

of very strong electrostatic effects, might even be almost completely insensitive to ionic strength within the physiological range. However, in the present case it seems that ionic strength changes should result in experimentally measurable changes in the kinetics of the MDH−CS protein.

This aspect leads us to consider how contact between our theoretical findings and experimental results is to be made; it is important to stress, for example, that there is as yet no direct experimental evidence that transfer of oxaloacetate is electrostatically mediated. One problem with connecting the present work to experimental results is the use here of pig heart enzyme structures, when the experimental work involved the yeast forms of the enzymes. As stated in the introduction, this difference is unavoidable owing to the lack of structural information for the yeast enzymes. A related difficulty involves our overall structure of the fusion protein; in the absence of further structural information we have no way of knowing whether our structure represents a good or poor model of the real fusion protein. Before dismissing the results reported here as artifacts of our modeled structure however, it should be borne in mind that it places the MDH active site facing away from CS and thus would not be expected to bias the results toward very efficient channeling. A more fundamental problem, and one of obvious importance, is how to connect the transfer efficiencies obtained by simulation with lag times and other observables obtained by experimental kinetics studies. Preliminary results (unpublished work) based on a reaction kinetics scheme suggest that the transfer efficiency of ∼45% obtained in our simulations is probably sufficient to account for the channeling of oxaloacetate observed in the AAT competition experiments (Lindblah *et al*., 1994). The development of a more complete method for connecting theoretical and experimental results, which will clearly be essential for example for interpreting ionic strength effects, will form the subject of further work.

Irrespective of how directly our findings can be compared to experimental results, the 100-fold increase in transfer efficiency obtained by changing the substrate charge from 0 to −2e in our simulations argues strongly that electrostatics may be of importance for the MDH−CS system. It is interesting therefore to ask whether such a mechanism is also likely to operate elsewhere in the citric acid cycle, particularly in view of the fact that all of the cycle's substrates (intermediates) have a charge of −2e or −3e. The existence of a multienzyme complex (metabolon) formed by enzymes of the citric acid cycle has been known for some years (Barnes & Weitzman, 1986), though it remains poorly characterized (Robinson *et al*., 1987). From a simulation point of view, interesting extensions of this work would involve investigating the two enzymes bracketing MDH and CS in the citric acid cycle, namely, fumarase and aconitase. High-resolution structures are available for both enzymes, but further information would be required before further theoretical work would be justified: in the absence of additional structural constraints, the large number of possible relative orientations of the enzymes would preclude meaningful study.

While the options for further theoretical study of channeling effects in multienzyme complexes are limited by the requirement for good structural information, experimental studies, such as kinetics measurements, suffer from no such limitation. However, in the absence of structural data, the *interpretation* of the experimental results may well be difficult. It is important to point out for example that in studies in which two enzymes, which would otherwise remain separate, are forced to associate [for example by the use of a covalent linker as in the work of Lindblah *et al*. (1994)] it may not be possible to decide with certainty whether the absence of a channeling effect is real or simply due to the fact that structural constraints force the enzymes to adopt a relative orientation unfavorable for channeling. By the same token, the efficient channeling obtained in the MDH−CS fusion protein might conceivably result from the enzymes being forced to adopt a relative orientation which just happens to be favorable for channeling. In the citric acid cycle metabolon itself, the two enzymes will probably be oriented in a different way, which may either increase or decrease the efficiency of channeling.

Finally it is worth pointing out that, even if accurate structural data are available, the simulation results reported here indicate that simple inspection of a structure may not be a good guide to estimating the efficiency of substrate channeling. If an electrostatic mechanism is important, as is suggested strongly by the present results, the spatial proximity of active sites need not correlate at all with the substrate transfer efficiency; what is likely to be more important is the presence of an unbroken electrostatically favorable region connecting the active sites.

ACKNOWLEDGMENT

A.H.E. is very grateful to Drs. Debbie L. Benson and Gary A. Huber for valuable discussions.

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BI9614747