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Model of a Quinary Structure between Krebs TCA Cycle Enzymes: A Model for the Metabolon†

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ABSTRACT: The enzymes which are responsible for catalyzing sequential reactions in several metabolic pathways have been proposed to be highly organized in supramolecular complexes termed metabolons. However, the *in situ* existence of these weak complexes is difficult to demonstrate because many of them are dissociated during isolation due to dilution effects. Consequently, the metabolon concept is subject to controversy. A model system consisting of genetically prepared bienzymatic fusion proteins has been used to immobilize sequential metabolic enzymes in close proximity and to demonstrate possible kinetic advantages of metabolons. These experiments use the sequential Krebs TCA cycle enzymes from yeast mitochondrial malate dehydrogenase (MDH), citrate synthase (CS), and aconitase (ACO). Using the porcine high-definition structures of these three enzymes, we have performed computer-modeling studies in order to understand how the molecules may interact. Among the thousands of docking orientations we have tried, one was found to respond to the structural and experimental constraints from the results obtained with the yeast fusion proteins. Interestingly, this quinary structure model shows substantial interacting surface areas with spatial and electrostatic complementarities which make the complex thermodynamically stable. This structure also contains an unbroken electrostatically favorable channel connecting the active sites of ACO and CS, as well as the one previously reported between CS and MDH active sites. Charged amino acids which could be involved in interactions stabilizing the complex have been identified. This model will be used as the basis for further experimental work on the structure of the Krebs TCA cycle metabolon.

Over the last 20 years, a considerable body of evidence supports the idea that sequential enzymes within a metabolic pathway interact with each other to form highly organized enzyme complexes (see ref 1 for review). The term metabolon was introduced (*2*) to describe such supramolecular complexes of sequential metabolic enzymes.

Data from a number of experimental approaches support the metabolon concept in connection with the Krebs TCA[1] cycle, demonstrating specific interactions between six of the eight sequential enzymes, including malate dehydrogenase (MDH), citrate synthase (CS), and aconitase (ACO) (see ref 3 for review). Moreover, it was found that all of the enzymes, previously thought to exist in a soluble form in the mitochondrial matrix, bind to the inner surface of the mitochondrial inner membrane, while purified isozymes from other cellular compartments do not possess such binding abilities (see ref 3 for review). It was also demonstrated that five of the Krebs TCA cycle enzymes (fumarase, MDH, CS, ACO, and isocitrate dehydrogenase) from *Escherichia coli* can be isolated as a high molecular weight complex able to catalyze the sequential reactions leading from fumarate to α-ketoglutarate (*4*).

A challenging question to be asked is "What are the advantages for the cell of this putative enzyme organization when compared to a system where the cell is a bag containing all the enzymes free in the bulk solution?". One possible

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[1] Abbreviations: ACO, aconitase; Aco1p, yeast mitochondrial aconitase; CS, citrate synthase; CS1, yeast mitochondrial citrate synthase; MDH, malate dehydrogenase; Mdh1p, yeast mitochondrial malate dehydrogenase; mMDH, pig mitochondrial malate dehydrogenase, cMDH, pig cytosolic malate dehydrogenase; TCA cycle, tricarboxylic acid cycle.

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reason for the organization is to prevent the intermediates from escaping into solution where they may be sequestered by other enzymes for use in different metabolic pathways (*5*, *6*). In other words, the close proximity of enzymes responsible for catalyzing consecutive steps of a metabolic pathway may be used to increase the metabolic flow through this pathway, by assuring the channeling of the intermediate. This proposition was examined with different techniques, and all of them gave consistent results supporting this notion of channeling (see ref 3 for review).

Despite the fact that more and more experimental data support the metabolon concept and establish unequivocally that enzymes are not free in the cell, one sees a continued implicit use of the notion of free enzymes when all aspects of cellular activities are discussed. This is a simplistic view of metabolism which is not without consequence since it has been amply demonstrated that the kinetic behavior of enzymes free in solution, upon which many of our concepts of metabolic regulation are based, is quite different from their behavior when they are bound. What experimental approaches might be considered to increase and/or to confirm the available data concerning the organization of metabolic pathways? To study the effects of close proximity between sequential metabolic enzymes, we have recently used a model where these enzymes are genetically fused by molecular biological techniques. This was successfully performed with yeast mitochondrial CS (CS1) and MDH (Mdh1p) (*7*, *8*) and recently has been extended to ACO (Aco1p). However, it is clear that the real demonstration for the existence of metabolon will require structural information about these supramolecular complexes. From this viewpoint, these fusion proteins are very attractive since high-resolution structures are available for these three enzymes, and modeling studies can provide insight into the way these proteins may interact and how metabolite intermediate transfer can be channeled between the active sites of sequential enzymes. For instance, a recent study of the CS1-Mdh1p fused protein (*9*) indicates that an electrostatic channel between the two active sites could explain the observed kinetic results (*8*). Elcock and McCammon (*9*) studied our structure by Brownian dynamics simulations and their calculations showed an electrostatic channeling between the CS and MDH active sites.

We report here computer graphic modeling studies which illustrate a possible docking orientation between the pig heart mitochondrial forms of ACO, CS, and MDH. This fitting is compatible with the previous model (*8*, *9*) of the docking between CS and MDH and provides evidence for the existence of an electrostatically favorable channel to guide citrate between the ACO and CS active sites. The electrostatic profile of the docking surfaces between ACO and CS and between CS and MDH enabled us to identify charged amino acids which may be involved in electrostatic interactions between the corresponding enzymes.

MATERIALS AND METHODS

Modeling Studies. The modeling studies have been performed using the molecular modeling system INSIGHT II (*10*). The crystal structures of pig mitochondrial MDH (*11*), pig CS (*12*), and pig mitochondrial ACO (*13*) were used to generate a model of docking orientation between these three enzymes. The "open" form of CS was used as it has been suggested to represent a substrate entry/product release form of the enzyme (*12*). The crystal structure of this form of CS was obtained with citrate bound and, therefore, is supposed to represent the best conformation to study the existence of an eventual channel connecting the active sites of ACO and CS. Bound citrate molecules were removed from both the MDH and CS structures, and bound isocitrate molecule was removed from ACO. The crystal structure of pig cytosolic MDH (*14*) was used for comparison studies with mitochondrial MDH. Hydrogens were added to each enzyme using the molecular simulation program X-PLOR (*15*).

Molecular Surfaces and Electrostatic Potentials. The building of molecular surfaces as well as the calculation of the electrostatic potentials were carried out with the program GRASP (*16*). Titrable 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. All histidines were in their neutral form. The total charge on the mitochondrial MDH dimer was therefore +6e, that on the CS dimer was −2e, that on ACO was 0e, and that on cytosolic MDH was −6e. The ionic strength was set to 0 mM in all the calculations.

Identification of the Charged Amino Acids of the Docking Surface. The molecular surfaces were built and the electrostatic potential was calculated for each molecule independently of the other enzymes. Residues that contributed most clearly to the electrostatic profile of the molecule were selected and then subjected to second round of inspection in INSIGHT II (*10*). Only those residues that could potentially pair with a complementary residue of the opposing face to form a charge−charge interaction were included in the final list of residues most likely to influence the selectivity of the interaction between the two enzymes.

RESULTS AND DISCUSSION

Given the experimentally derived constraints of C-terminus CS docking with N-terminus MDH and C-terminus ACO docking with N-terminus CS, a molecular model was simply derived which showed (1) large interaction surfaces which are spatially and electrostatically complementary and (2) electrostatic channels between the sequential active sites.

Model of Interaction between ACO, CS, and MDH. Since a docking orientation between the carboxy-terminus of CS and the amino-terminus of MDH was already found to be favorable (*8*, *9*), the docking between CS and ACO was performed in the presence of MDH so as to exclude from the interactions with ACO the CS surface which is proposed to interact with MDH. The problem of docking two free enzymes together in some reasonable fashion is a difficult one with probably many solutions, but the search for a likely orientation of ACO with respect to CS was greatly facilitated by the existence of different experimental and structural constraints. First, CS exists as a constitutive homodimer; thus, most of the monomer surface area is already occluded in an interaction with another CS molecule. Second, the 2-fold symmetry of the CS dimer interaction makes it likely that two ACO molecules bind to the surface of CS in such a way as to preserve the symmetry of the complex. Moreover, to construct the fusion enzymes between ACO and CS, the gene encoding the ACO molecule is fused with the gene encoding one CS monomer, so that the formation of the CS dimer can still occur in the presence of two ACO molecules in the complex. Thus, each possible orientation

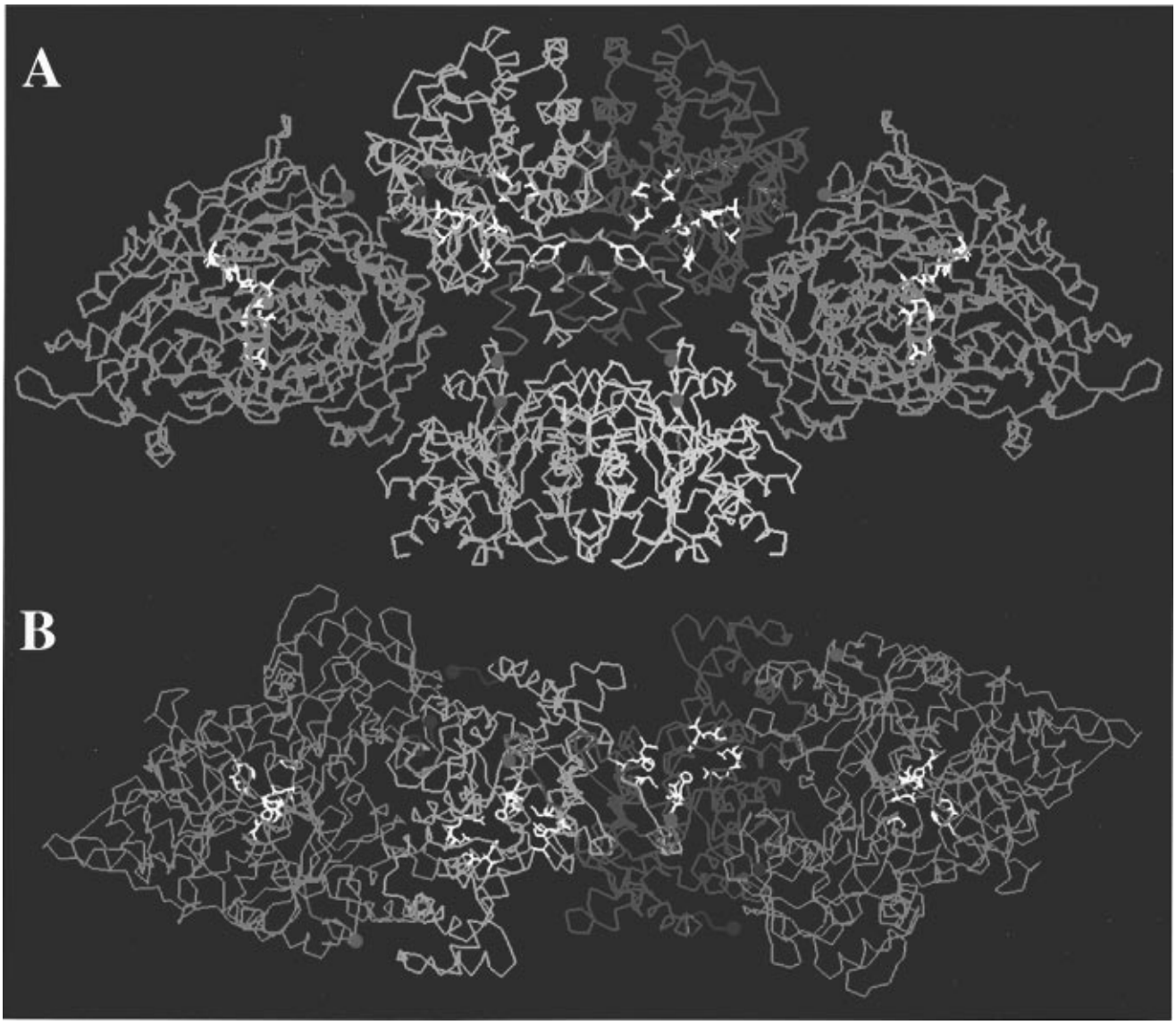

FIGURE 1: Graphic simulation for the pig mitochondrial MDH, CS, and ACO interactions. (A) View of the complex showing the final docking orientation between ACO, CS, and MDH. Only the Cα trace of each enzyme is shown. The two monomers of CS are represented in red and orange, with, for each monomer, the first four N-terminal residues colored in green and the last four C-terminal residues colored in pale blue. ACO molecules are represented in blue with the first four C-terminal residues colored in green. The two monomers of MDH are represented in bright and pale pink, with the first four N-terminal residues of each monomer colored in pale blue. The free extremities fused in our fusion proteins are shown by a spot. The active sites of ACO and CS are indicated in white. (B) Complex in panel A without MDH and rotated by 90° on a horizontal axis so that MDH would appear foreground. This figure was prepared using INSIGHT II (*10*).

of ACO with CS was evaluated with the understanding that the complex might very possibly accommodate two molecules of ACO for every one CS dimer. Third, another consideration which presented itself in the construction of the model was information regarding the flexibility of several regions of the ACO crystal structure. Two regions in particular, 522−527 and 752−754, were flagged in the PDB file (7acn) to indicate that the data for these regions was particularly poor and reflected the fact that the protein structure in these regions is conformationally labile. Interestingly, in the final docking arrangement, these regions flank two opposite sides of the ACO/CS interface and may undergo some conformational rearrangements to secure the two enzymes in a stable complex. Fourth, in our experimental work, we used a very short linker region (three amino acids) to connect the carboxy-terminus of Aco1p with the amino-terminus of CS1, or vice versa. Consequently, pig CS and ACO enzyme structures were docked so that one of the pair of extremities connected together in the corresponding yeast fusion proteins remained in a proximity compatible with the distance spanned by three amino acids. Altogether, these requirements drastically reduced the orientational possibilities, and one docking orientation with minimal structural overlap seemed most favorable (Figure 1).

An overwhelming feature of the docking interaction as we have modeled it is the existence of a striking groove that forms a continuous channel on the surface of the complex and joins the active sites of ACO and CS (Figure 2A). Among the many docking orientations we have tried, we could find no other docking arrangement that presented such a clearly favorable surface topology to support the notion of assisted transfer of substrates between the active sites of ACO and CS, while adhering to all the above-cited structural and experimental constraints. The width of this groove (12 Å in the narrowest part to 29 Å in the widest part) is sufficient to accommodate a citrate molecule. Moreover, from calculation of the electrostatic potential around the complex, the groove appears to be continuously positively

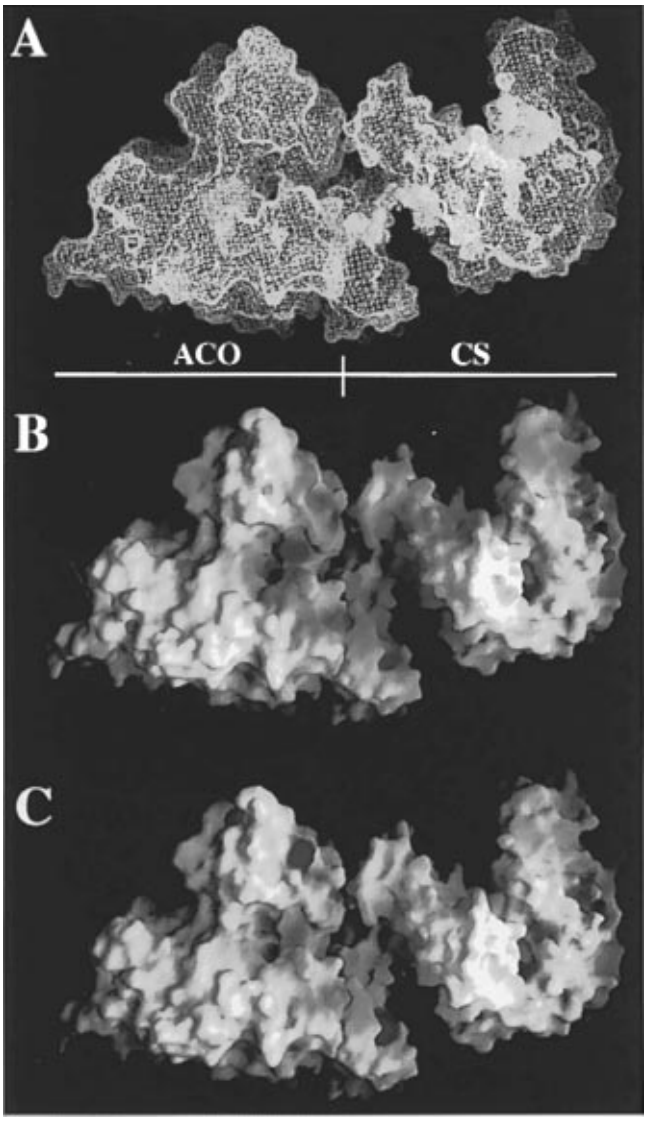

FIGURE 2: Surface of ACO−CS complex model viewed in a similar position to that in Figure 1B. Only one ACO molecule and the CS dimer are shown. (A) The molecular surface is drawn as a mesh grid. Atoms of the active site residues from each enzyme are represented by space-filling spheres and colored green for ACO and yellow for CS. (B) The molecular surface shown in panel A is colored according to the electrostatic potential calculated at zero ionic strength and in presence of MDH. Blue areas represent electrostatically positive regions; red, electrostatically negative regions (limits set at ±15 kT). (C) Same as panel B except that the electrostatic calculation was performed in absence of MDH. This figure was prepared using GRASP (*16*).

charged (blue) (Figure 2B). Citrate being negatively charged (−3e), this unbroken deep groove connecting the active sites represents an electrostatically favorable channel for guiding this intermediate transfer between these two Krebs TCA cycle enzymes. Since the electrostatic potential is extremely dependant on close environment, the electrostatic profile shown in Figure 2B was obtained in the presence of MDH (which, of course, was removed to visualize the groove) to mimic the channel environment within the metabolon complex. Interestingly, the calculation of the electrostatic potential around the ACO-CS surface complex in the absence of MDH gave a different electrostatic picture with some negatively charged areas (red) inside the groove (Figure 2C), suggesting that MDH is required for an efficient electrostatic channeling of citrate between the active sites of ACO and CS.

As seen in Figure 1, this model contains a close proximity (12.5 Å) of the ACO carboxy-terminus and CS amino-terminus (in green), while the two other extremities (pale blue) are maintained at a distance from each other (51 Å). Interestingly, the preliminary results obtained with fusion proteins of Aco1p and CS1 indicate that CS activity is 4−5 times higher from the fusion protein with Aco1p in the N-terminal part than from the reverse one (unpublished results). The distance spanned by a sequence of three amino acids is less than 12.5 Å but, in the CS dimer structure shown in Figure 1, the first four N-terminal residues of each monomer are missing. Consequently, in the corresponding fusion protein the 12.5 Å distance gap found in the model between the two closest termini is spanned by seven amino acids and could easily be accommodated in the folding of the fusion protein. The three enzyme fusion proteins consisting of Aco1p C-terminus fused with CS1 N-terminus and CS1 C-terminus fused with Mdh1p N-terminus (with a three amino acid linker in each case) is functional (unpublished results), which is also in agreement with this model of the complex. In this supramolecular complex model, the distance between the ACO and CS active sites is about 45 Å. This is such a relatively large distance and it would appear at first sight unlikely that substrate would be transferred between active sites with much efficiency. However, Elcock and McCammon (*9*) performed Brownian dynamics simulations to investigate the channeling of oxaloacetate in the CS-MDH fusion protein and provided evidence for an efficient electrostatically based channeling mechanism, even though MDH and CS active sites are separated by nearly 60 Å in the model of the fusion protein used for this study. Moreover, according to these simulations, it is likely that, in the absence of electrostatic effects, only a few substrate molecules leaving the CS active site would reach the ACO active site. The total solvent accessible surface area buried between the ACO and CS upon formation of the complex (7500 Å^2) as calculated in GRASP (*16*) is quite substantial. Previously, an analysis of crystal structures of known protein−protein interactions and antigen/antibody complexes was performed (*17*) and led the authors to suggest that most protein−protein interactions that form stable complexes *in vitro* bury on the order of 2000 Å^2 or more of total surface area in an interaction. The large surface area buried in the ACO/CS docking arrangement is compatible with their results and suggests that the complex as we have modeled it might indeed be thermodynamically favorable. Moreover, the central core of the interface is represented in our model by a relatively flat surface that contains a mixture of polar and hydrophobic residues; both types of residues are characteristics of an interface that is thermodynamically stable and yet able to engage only specific molecules.

Electrostatic Studies of the Docking Surfaces. To study the interacting surfaces of the proteins in the complex and the existence of possible electrostatic forces which could attract the proteins together and stabilize the complex, we next examined the electrostatic potential of each docking surface (Figure 3). The identification, in GRASP (*16*), of the charged amino acids from the putative docking sites was performed as described in Materials and Methods. Using INSIGHT II program (*10*), it was possible to locate these residues (by coloring them) in the complex shown in Figure 1A and determine which could be involved in electrostatic

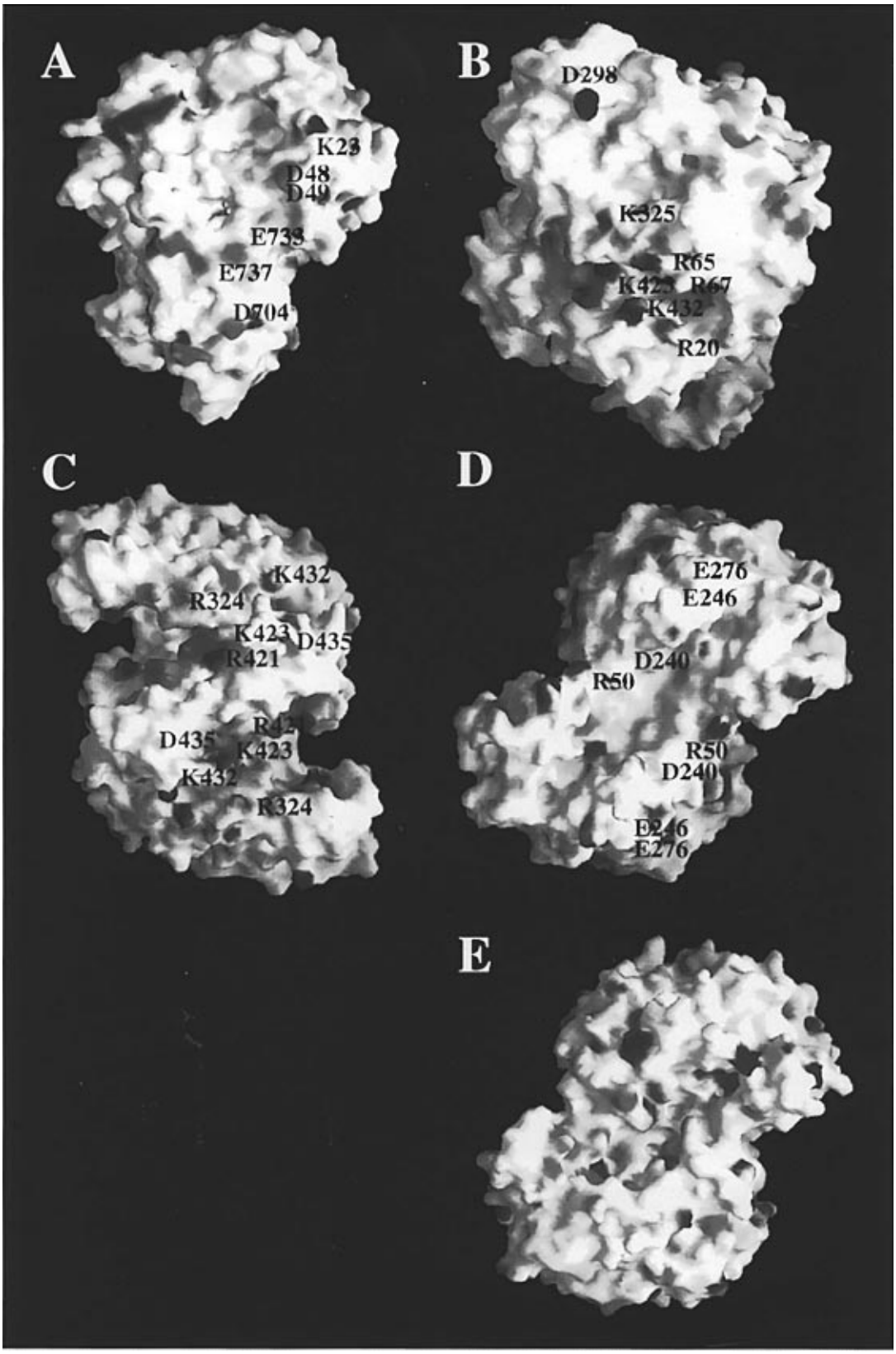

FIGURE 3: Docking surfaces of ACO (A) and CS (B) and of CS (C) and MDH (D), according to the complex model shown in Figure 1. These surfaces are represented so that the left part of the surface shown in the left panel docks with the right part of the surface shown in the right panel and that the tops of both surfaces dock together (the two surfaces roll up one on the other as the closing of a book). The molecular surfaces are colored according to the electrostatic potential as described in Figure 2B. The amino acids indicated (using the "one letter" nomenclature) are those thought to be involved in electrostatic interactions between the two molecules and were identified as described in the text. As shown in Figure 1A, the docking surfaces between CS and MDH involve the two monomers of each enzyme and it is the reason why each amino acid is indicated twice on panels C and D. (E) Electrostatic surface of cMDH viewed from an orientation similar to that of its counterpart mMDH shown in panel D. This figure was prepared using GRASP (*16*).

interactions with complementary charged amino acids of the other molecule. The electrostatic profiles of the docking surfaces between ACO and CS are shown in panels A and B of Figure 3. Those charged residues of ACO which could

undergo conformational changes using the standard library of side chain rotamers in INSIGHT II (*10*) and occupy positions that would put them within 4 Å of a complementary charged residue on the opposing surface of CS were selected as possible interacting amino acid pairs in the complex: ACO Lys 23 with CS Asp 298, ACO Asp 48 or Asp 49 with CS Lys 325, ACO Asp 704 with CS Arg 20 or Lys 432, ACO Glu 733 with CS Arg 65 or Lys 423, and ACO Glu 737 with CS Arg 67. Other charged residues were identified in these putative docking surfaces but probably do not participate in the electrostatic interactions between the two proteins because of the absence of a charged partner in close proximity on the other surface and because they are involved in a salt bridge with an adjacent complementary charged amino acid in the same polypeptide chain. The same type of analysis was performed for the docking surfaces between CS and MDH. As shown in panels C and D of Figure 3, the center of the CS dimer docking surface is mainly positively charged (blue) while the MDH dimer one is mainly negatively charged (red). However, only a few pairs of possible interactions have been identified without ambiguity (CS Asp 435 with MDH Arg 50, CS Arg 324 or Lys 432 with MDH Glu 246 or Glu 276, and CS Arg 421 or Lys 423 with MDH Asp 240 or Glu 246), because many of the acidic or basic amino acids which contribute to the global charge of these docking surfaces (i.e., to the red and blue spots) proved to be slightly buried in the molecule and not structural components of the surface. Consequently, these other residues may contribute to the electrostatic forces responsible for attracting the two molecules together but could not be involved in direct electrostatic interactions stabilizing the complex.

Since the metabolon concept emphasizes that the specific binding ability of the outer surface of an enzyme is as important as the enzyme activity itself, the structural comparison between isoforms which take part in different pathways is crucial to investigate the metabolon. There is no extramitochondrial CS in mammals, and the pig cytosolic aconitase structure has not been solved, but a high-resolution structure is available for pig cytosolic MDH. In spite of a considerable difference in amino acid sequence between pig mitochondrial MDH (mMDH) and cytosolic MDH (cMDH) (only 10% amino acid sequence identity), the structural similarity is high and both enzymes show the same global shape (compare panels D and E of Figure 3). In this regard, Ian Burbulis has shown, using a different docking program (QUANTUM, version 4.0), that both pig heart cMDH and mMDH dock equally well with CS in the same conformation as shown here (personal communication). Because of this large difference in amino acid composition between cMDH and mMDH, there is a strong divergence between the electrostatic profile of the surface of mMDH that is proposed to dock with CS and the one of the corresponding surface of cMDH (compare panels D and E of Figure 3). Moreover, none of the mMDH residues proposed to be involved either in electrostatic interactions with complementary charged amino acids of CS or in the contribution of the global charge of the docking surface (see above) are conserved in the sequence of cMDH. These data suggest that cMDH is not able to replace its mitochondrial counterpart in the Krebs TCA cycle metabolon and reinforce the idea that isoenzymes could differ from each other by their binding specificity to other components of the cell, which is one of the corollaries of the metabolon concept.

The overwhelming feature of the model we describe here is the fact it complies, at one time, with our experimental constraints, with the criteria of a thermodynamically stable complex, and with the notion of electrostatic channeling in guiding substrate transfer between the enzymes. However, a problem remains in connecting the modeling work to our experimental studies. The enzyme structures used for these theoretical studies are from pig heart, the yeast ones being not available, but pig heart enzymes are expected to be good models for yeast enzymes and we can assume similar structures between the pig and the yeast fusion proteins. On the other hand, a related difficulty involves our overall structure of the fusion proteins; in the absence of further structural information, we have no way of knowing whether our structure represents a good or a poor model of our fusion proteins, even if they are performed with pig enzymes. In an attempt to elucidate the structure of these fusion proteins, we will use these theoretical data to construct the pig fusion enzymes and modify, by directed mutagenesis, the amino acid residues thought to be involved in electrostatic interactions between the proteins. These experiments are now in progress. If these charged amino acids are required to stabilize the complex, we can expect big differences in the channeling of the intermediates and kinetic parameters between the wild-type and the mutated fusion enzymes. Here is an illustration of how a theoretical modeling can be the starting point for further experimental study.

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