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Direct Evidence for Metabolon Formation and Substrate Channeling in Recombinant TCA Cycle Enzymes

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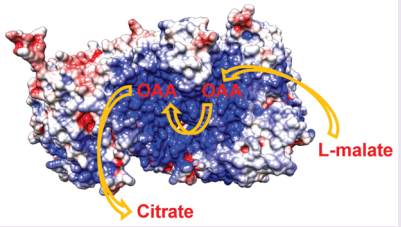
Supporting Information

ABSTRACT: Supramolecular assembly of enzymes into metabolon structures is thought to enable efficient transport of reactants between active sites *via* substrate channeling. Recombinant versions of porcine citrate synthase (CS), mitochondrial malate dehydrogenase (mMDH), and aconitase (Aco) were found to adopt a homogeneous native-like metabolon structure *in vitro*. Site-directed mutagenesis performed on highly conserved arginine residues located in the positively charged channel connecting mMDH and CS active sites led to the identification of CS(R65A) which retained high catalytic efficiency. Substrate channeling between the CS mutant and mMDH was severely impaired and the overall channeling probability decreased from 0.99 to 0.023. This work provides direct mechanistic evidence for the channeling of reaction intermediates, and disruption of this interaction would have important implications on the control of flux in central carbon metabolism.



Enzymes frequently function in sequential, multistep cascades, and the colocalization of the enzymes in self-assembling clusters is often observed.[1−3] The term "metabolon" has been used to describe these noncovalent dynamic enzyme complexes.[4] These arrangements enable substrates to be channeled between active sites without escaping into the medium.[5] When the intermediate transport is not 100% efficient, leaky channeling can occur, but the intermediates are sequestered enough to prevent equilibrium with the surroundings.[5−7]

Substrate channeling within metabolons results in several metabolic advantages. High local substrate concentrations enable better fluxes through a pathway, despite unfavorable equilibrium constants. Intermediates can be protected from the bulk phase, hindering competition from alternative pathways and protecting the cell from toxicity. These effects on the mass transport allow the enzymes to operate at high efficiencies even when the average concentrations of intermediates in the bulk phase are low, resulting in the improvement of overall catalytic efficiency of the metabolic process.[6−10]

Metabolons exist in many pathways, including glycolysis, fatty acid oxidation, amino acid metabolism, lipid biosynthesis, and the tricarboxylic acid (TCA) cycle.[5,11] Several enzymes of the TCA cycle participate in metabolon formation, including citrate synthase (CS), mitochondrial malate dehydrogenase (mMDH), and aconitase (Aco).[12−15] Since they play a central role in cellular energy generation, metabolons of the TCA cycle have been well-studied.[16−23] The CS/mMDH interactions are of particular interest since the free oxaloacetate (OAA) intermediate concentration in the cell is thought to be too low to sustain the experimentally determined cycle rate and the mMDH reaction has an unfavorable equilibrium constant in the forward direction of the cycle.[20,23]

Most metabolon investigations employ indirect techniques to infer channeling.[8,14,21] Fundamental characterizations of substrate channeling have focused on enclosed channels such as the tunneling that occurs in tryptophan synthase.[24−26] However, the bounded diffusion mechanism within the metabolon is more relevant to most biological systems. And, it is becoming clear that these "leaky channeling" systems are inspiring new approaches in biocatalysis where coupled reaction/transport systems are being engineered with biomimetic substrate channeling pathways.[7] Recently, the first structural characterization of a native mMDH−CS−Aco TCA cycle complex was reported.[27] In this work, we created and characterized recombinant versions of the TCA cycle enzymes, and this enables the use of site-directed mutagenesis to explore structural determinants of substrate channeling *in vitro*.

RESULTS AND DISCUSSION

The TCA cycle enzymes are a canonical example of the importance of substrate channeling. Characterizing the leaky channeling within this system has been difficult due to limited experimental tools. The first structural evidence for natural metabolon formation and subsequent electrostatic substrate channeling within these enzymes was recently obtained by resolving the three-dimensional structure of the mMDH−CS−Aco complex by *in vivo* chemical cross-linking, mass spectrometry, and protein docking.[27] Here, we characterize a

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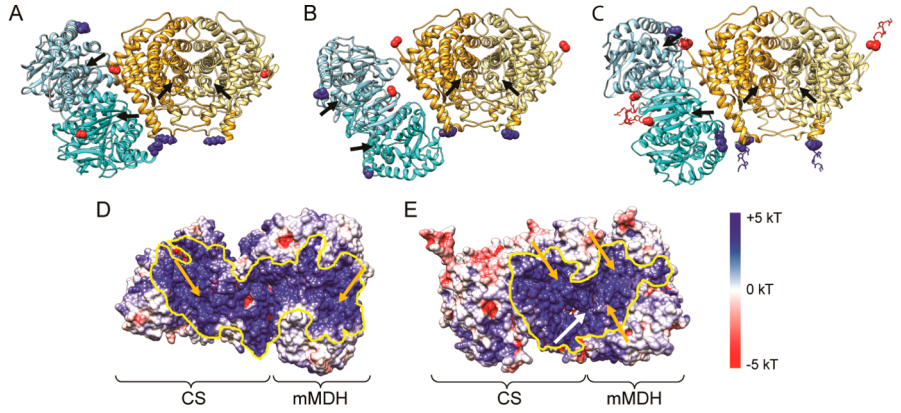

Figure 1. Structures of the mMDH−CS complex and the simulated electrostatic potential on the surface of the complex. (A) Native tissue mitochondrial metabolon. (B) *In vitro* complex formed by commercial enzymes. (C) *In vitro* complex formed by recombinant enzymes. N- and C-termini are represented by red and blue spheres, respectively. FLAG-tag and polyhistidine-tag are represented by red and blue sticks, respectively. mMDH and CS active sites are denoted by black arrows. (D) *In vitro* complex formed by commercial enzymes. (E) *In vitro* complex formed by recombinant enzymes. Surface regions of positive potential and negative potential are colored in blue and red, respectively. The electrostatic channeling path for OAA is highlighted by the yellow edge. Orange arrows indicate the active sites. The white arrow indicates the location of Arg65 and Arg67. The surface ESP was calculated with water molecules at pH 7.4.

synthetic metabolon formed *in vitro* by three recombinant versions of these enzymes of the cycle, which form a similar conformation to that of the natural complex *in vivo*. Substrate channeling is further investigated by site-directed mutagenesis, and channeling can be significantly impaired by a single site-directed mutation.

The mMDH and CS enzymes are dimers composed of identical subunits, weighing 34 kDa and 49 kDa respectively, whereas the next enzyme, Aco (85 kDa), is monomeric.[28−30] mMDH catalyzes the reversible NAD(H)-dependent conversion of L-malate and OAA. CS converts OAA and acetyl coenzyme A (acetyl-CoA) to citrate and coenzyme A (CoA). Aco catalyzes the dehydration−rehydration of citrate to isocitrate, with *cis*-aconitate being the intermediate. These enzymes have been individually characterized, with porcine heart being the most extensively studied variants, which share >95% sequence homology with bovine heart enzymes. Here, three different enzyme groups were investigated: native tissue enzymes isolated from the intact bovine mitochondria, commercially available porcine wild-type enzymes, and recombinantly produced porcine enzymes. For the recombinant enzymes, codon optimized synthetic genes coding for porcine heart mMDH, CS, and Aco were expressed in *E. coli*. Enzymes were purified to >90% (Figure S1), and the protein yields were 5 mg L^{-1}, 75 mg L^{-1}, and 50 mg L^{-1} for mMDH, CS, and Aco, respectively.

***In Vitro* Metabolon Conformations.** Over the past decade, cross-linking/mass spectrometry analysis has been a common and standardized technique for studying protein complexes that cannot be evaluated by X-ray or NMR analysis.[31,32] *In vitro* chemical cross-linking of protein−protein interactions between recombinant mMDH and CS was performed in the presence of Aco. SDS-PAGE analysis of commercial and recombinant enzyme mixtures demonstrated the formation of higher-ordered complexes after incubation with disuccinimidyl glutarate (DSG), indicated by a set of intense bands above 100 kDa (Figure S2). In-gel tryptic digestion was conducted on the large complex bands, and the extracted peptide fragments were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

In the mitochondrial matrix, compartmentalized TCA cycle enzymes diffuse slowly, and their dynamic association is stabilized by the crowded environment. On the contrary, the apparent diffusion coefficient of enzymes in dilute solution is approximately 2 orders of magnitude higher, and the random molecular collision occurs much faster[11,33−36] than DSG cross-linking chemistry, so it is not possible to isolate and purify a stable *in vitro* complex without cross-linking, and there are minimal experimental artificats from random molecular collisions without strong intermolecular interactions. However, the *in vitro* system only contains the three enzymes (mMDH, CS, and Aco), so the mass spectrometric data analysis and protein docking are easier, because the identities of all of the enzymes participating in the complex are known. Matching experimentally detected tryptic peptides to protein databases using the *Mascot* search engine identified the three enzymes in both cross-linked and non-cross-linked sample bands (Table S1). Cross-linked peptide candidates were determined by comparing cross-linked and non-cross-linked mass spectra and matching additional masses after cross-linking to a manually built theoretical mass database. Using the distance restraint (25 Å) on potentially DSG-linked residues (Figure S3), a hybrid protein docking method was utilized to elucidate the interactions between mMDH and CS *in vitro*, as previously described.[27]

For the complexes of commercial enzymes, a number of structures were found to meet the selection criteria and bear at least three identified cross-links (Figure S4). These structures exhibited distinct conformations, implying that mMDH and CS without any modifications interact in a random manner in dilute solution. Compared to the native tissue mitochondrial TCA cycle metabolon (Figure 1A), one model of the complex of commercial mMDH−CS showed up with the most structural similarity. In this structure (Figure 1B), three DSG cross-links (matching three MS peaks, Table S2) were obtained between mMDH Lys191, Lys277, Lys283, and CS Lys325. The α-helices of CS at Ala1-His28 and Ser426-Lys437 are buried in the interface that covers the intersubunit domain of mMDH. Compared to the native tissue mitochondrial metabolon *in vivo*, however, mMDH is flipped around the axis parallel to the binding interface by about 180°. As a result, the two N-termini

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of mMDH are in close proximity with CS while the C-termini are pointing outward. This flipping may be less favorable for channeling of OAA, as the mMDH active site clefts are open to the bulk phase and separated from CS active sites by a longer distance (73 Å) than that in the native tissue mitochondrial metabolon (35 Å).[27]

In contrast to the random association of commercial enzymes that yielded a number of complex conformations, the recombinant enzyme interactions were more restricted and resulted in a unique structure bearing seven DSG cross-links (matching four MS peaks, Table S3) between mMDH Lys81, Lys217, Lys304, and Lys305 and CS Lys76, Lys325, and Lys432. The α-helices of CS near its N- and C-termini again participate in the binding interface with the intersubunit region of mMDH. Although mMDH was rotated around the axis perpendicular to the interface by approximately 30° as compared to the metabolon *in vivo*, the final structure maintains most of the natural features (Figure 1C). No termini are buried in the interface, possibly due to spatial hindrance from additional amino acids not present in commercial or native mitochondrial enzymes appended to the termini (FLAG-tag (eight amino acids on N-terminus) and polyhistidine purification tag (6 amino acids on C-terminus)), but the relative locations of termini around the interface are not significantly altered. The two N-termini of the mMDH dimer point away from CS, and the mMDH and CS active sites are brought within a closer proximity (40 Å) than what was observed in the complexes of commercial mMDH−CS (73 Å). With this shorter pathway, OAA transfer was expected to be faster than that in the wild-type complexes. In addition, the interfacial areas in the recombinant mMDH−CS complexes are about 12 100 Å^2, suggesting that they may be more thermodynamically stable than either the native tissue mitochondrial metabolons (10 000 Å^2)[27] or the complexes of commercial mMDH−CS (11 300 Å^2). Of course, these measurements are based on docking of rigid protein models, and subtle conformational changes in the proteins upon self-assembly could lead to changes in the interaction areas.

Formation of commercial and recombinant mMDH−CS−Aco complexes *in vitro* was also examined by docking Aco onto solved mMDH−CS complexes (Figure S5). Three DSG cross-links (matching three MS peaks) were identified between CS Lys16, Lys76, and Lys80 and Aco Lys4 and Lys117 in the trienzyme complex formed by commercial enzymes (Table S4). It was found that CS Lys76 and Lys80 were located in the mMDH−CS interface, implying that mMDH and Aco would not be present on the same subunit of CS. Therefore, each CS dimer can only bind one mMDH dimer and one Aco monomer (Figure S5). In the recombinant mMDH−CS−Aco complexes, there were four DSG cross-links between CS Lys294 and Lys300 and Aco Lys709 and Lys712, exhibiting no conflict with the identified cross-links between mMDH and CS, although only one MS peak was matched (Table S5). This result is consistent with that obtained from *in vivo* cross-linking of CS and Aco. Residues within the C-terminal region of the recombinant Aco were recognized at the CS−Aco interface. A groove formed between mMDH and CS appeared to accommodate the C-terminus of Aco (Figure S5). Hence, the resulting trienzyme association was found to be more compact than that of the metabolon formed by commercial mMDH and CS. Similarly to the native tissue mitochondrial metabolon, a recombinant octamer comprised of one CS dimer, two mMDH dimers, and two Aco monomers could possibly form *in vitro*.[27]

A model of the three-enzyme system was built on the assumption that inclusion of Aco does not alter the complex formation between mMDH and CS. The interaction between Aco and CS (or mMDH) is weaker than that between mMDH and CS according to previous observations *in vivo*, even though there is a lower chance that CS (or mMDH) binds Aco prior to mMDH (or CS) in dilute solution. Therefore, the contribution of Aco to the structural assignment between mMDH and CS was of lesser interest, and the remainder of the experimental efforts were focused on the investigation of the channeling of OAA within the mMDH−CS complex, which is consistent with most of the related research in the literature.[17,19]

Simulated Electrostatic Channeling in Wild-Type/Recombinant Complexes. Elcock and McCammon previously demonstrated through Brownian dynamics simulations that electrostatic forces at the surface of a yeast mMDH−CS fusion protein greatly improved the OAA transfer efficiency.[19,37] In their fusion protein, a continuous surface of positive electrostatic potential bridged the active sites, implying an important role of surface charge in the directed transport of OAA. In the natural TCA cycle metabolon, theoretical evidence for electrostatic channeling was also found between mMDH and CS active sites using simulation tools.[27] To further investigate electrostatic channeling in the mMDH−CS complex formed *in vitro*, the electrostatic surface potential (ESP) was examined using the Poisson−Boltzmann equation in the presence of water molecules at pH 7.4. As illustrated in Figure 1D, a long and broad band of positive potential covers the majority of the complex surface of the commercial enzymes on one side, connecting the active sites. In the recombinant mMDH−CS complex (Figure 1E), the positive patch connecting active sites was reduced due to their increased proximity and relative orientation. Taken together with previous results from simulation of surface ESP of free enzymes, the formation of substrate channeling in such dynamic complexes is a product of electrostatic protein−protein interactions and rearrangement of surface charges upon association. Charged surface residues, especially positively charged arginines and lysines, likely play an essential role in directed transport of negatively charged OAA.

To explore this, interfacial residues of the recombinant complex were identified by screening surface arginine and lysine residues within a distance of 20 Å from each other. As a result, CS Arg65 and Arg67 were estimated to be important for the formation of the positive channel. Site-directed mutagenesis was performed at these positions, and six different CS mutants were explored, where Arg65 and Arg67 were replaced by either alanine or aspartic acid: R65A, R67A, R65A/R67A, R65D, R67D, and R65D/R67D. Prior mutational studies of CS have involved the active site residues,[38−41] aiming for the improvement of enzyme catalysis while the two residues mutated in this work (Arg65 and Arg67) are not located near the active site (Figure S6, Table S6).

Specific activities of the CS mutants were determined (Table S7). Arg67 was found to be crucial for the enzymatic activity of the recombinant CS. Any mutation of this side chain decreased or eliminated the enzymatic activity. In addition, CS(R67D) and CS(R65D/R67D) were found to be structurally affected by the mutations as shown in Figure S7. CS(R67A), CS(R65A/R67A), and CS(R65D) exhibited 2 or 3 orders of magnitude reductions of specific activities in comparison to the recombinant wild-type CS. CS(R67D) and CS(R65D/R67D) enzymatic activities could not be determined. CS(R65A) had

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similar enzymatic activity compared to recombinant wild-type CS. However, this mutation resulted in the dissociation of the mMDH−CS complex as implied by the disappearance of the mMDH−CS complex bands in native PAGE gels (Figure S8). Residues 65 and 67 were compared among different species, and Arg67 is highly conserved whereas Arg65 is generally well conserved, suggesting the importance of positive charge at these positions (Table S8).

The steady state kinetics of the recombinant mMDH, recombinant CS, and mutant CS enzymes were evaluated. Both mMDH and CS follow the ordered bi−bi kinetic mechanism (eq S1), where mMDH binds to its cofactor and CS binds to OAA first.[28,29] The full steady state kinetic parameters were determined for recombinant mMDH, CS, and CS(R65A) (Table 1). Recombinant CS had a $k_{cat}/K_{M,acetyl\text{-}CoA}$ value of 4.3

Table 1. Kinetic Parameters of Recombinant Enzymes[a]

enzyme	k_{cat} (s^{-1})	$K_{i,A}$ (mM)[b]	$K_{M,A}$ (mM)[b]	$K_{M,B}$ (mM)[c]
mMDH, fwd	31 ± 2	0.42 ± 0.08	0.13 ± 0.03	0.83 ± 0.10
mMDH, rev	870 ± 140	15 ± 2	87 ± 22	33 ± 7.9
CS	88 ± 4	4.6 ± 0.8	7.9 ± 1.2	21 ± 3
CS(R65A)	44 ± 3	1.7 ± 0.1	1.1 ± 0.3	7.8 ± 2.3

[a]Data are given as mean values ± SD from at least three independent measurements. [b]Substrate A is NAD^+ for mMDH, fwd; NADH for mMDH, rev; and OAA for both CS and CS(R65A). [c]Substrate B is L-malate for mMDH, fwd; OAA for mMDH, rev; and acetyl-CoA for both CS and CS(R65A).

μM^{-1} s^{-1} and $k_{cat}/K_{M,OAA}$ value of 11 μM^{-1} s^{-1}, whereas CS(R65A) had a $k_{cat}/K_{M,acetyl\text{-}CoA}$ value of 5.6 μM^{-1} s^{-1} and a $k_{cat}/K_{M,OAA}$ value of 39 μM^{-1} s^{-1}. These values indicate that R65A mutation did not impair the kinetic behavior of the enzyme.

Channeling in the Presence of a Competing Enzyme and in Viscous Solutions. A common method to probe substrate channeling is to introduce an enzyme competing for the same intermediate.[42] As illustrated in Figure 2A, the mMDH−CS complex catalyzes sequential conversion of L-malate to citrate via OAA as the intermediate, using NAD^+ and acetyl-CoA as cofactors. A competitive pathway was introduced with aspartate aminotransferase (AAT), which catalyzes the conversion of OAA and L-glutamate to aspartate and α-ketoglutarate. In the presence of AAT, the resultant rate of citrate generation measured with crude lysate containing the native tissue mitochondrial mMDH−CS complex showed little change (Figure 2B). Approximately 88% of the recombinant mMDH−CS activity was retained, whereas 72% of the commercial mMDH−CS activity was retained in the presence of 1 U mL^{-1} of AAT. As the AAT concentration was increased to 5 U mL^{-1}, 89%, 77% and 68% of the coupled activity remained in the native tissue mitochondrial, recombinant, and commercial enzyme complexes, respectively (Figure 2B). The mutant recombinant complex (mMDH−CS(R65A)) only retained 53% of the coupled activity for 5 U mL^{-1} AAT. To minimize potential mMDH−AAT interactions, coupled catalysis was also explored with complexes immobilized in modified chitosan polymers. This ensured that AAT in bulk solution was physically separated from the complex and its interaction with the metabolon could be minimized. Similar results were obtained compared to the free complexes in solution (Figure S9).

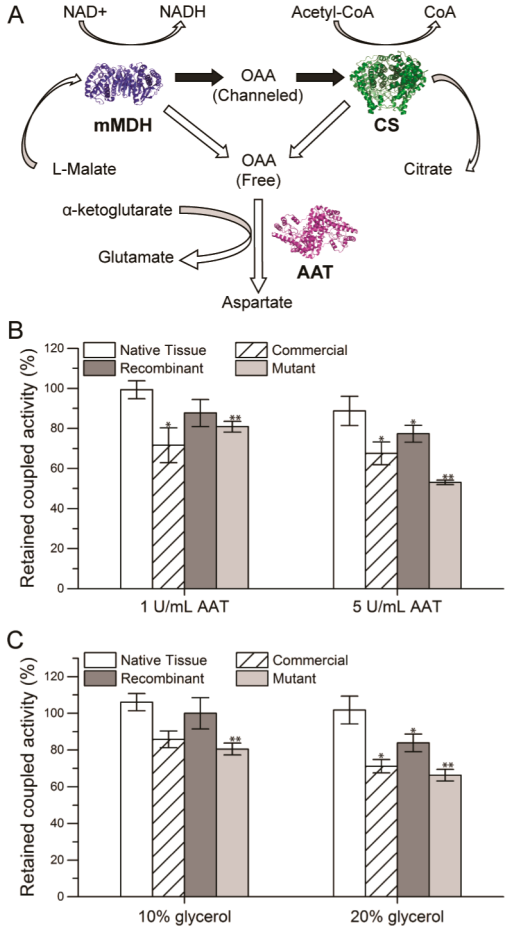

Figure 2. Demonstration of channeling of OAA within the mMDH−CS complex. (A) Schematic of the coupled mMDH−CS catalysis in the presence of competing enzyme AAT. White and black arrows represent diffusion and directed channeling, respectively. Crystal structure of AAT is obtained from Protein Data Bank (PDB ID: 1AAT). (B) Coupled activity retention calculated as the ratio of reaction rate before and after adding AAT measured with 100 μM mMDH−CS complex or crude mitochondrial lysate (about 5 μM protein in total). (C) Coupled activity retention in the presence of glycerol. Error bars represent standard deviation calculated from three independent experiments. Statistical significance (p value) with respect to samples without AAT was calculated by a two-sample t test with Welch correction for unequal variances: $*p < 0.05$, $**p < 0.01$.

The mitochondrial matrix is highly viscous, which will decrease mass transfer in the absence of substrate channeling. Glycerol was added into the assay solution in order to explore a more viscous environment, and the efficiencies of mass transport in different mMDH−CS complexes were compared. As Figure 2C demonstrates, coupled activity of the native tissue mitochondrial mMDH−CS complex in crude lysate was not affected by the increased viscosity. For the recombinant complex, catalysis was similar in 10% (v/v) glycerol, but decreased by 16% in 20% glycerol. This indicates that substrate channeling in the artificial complex functions in a "leaky" fashion. When compared to the complex of commercial enzymes and the mutant recombinant complex (mMDH−CS(R65A)), which respectively lost 15% and 20% of coupled activity in 10% glycerol and 30% and 35% of coupled activity in 20% glycerol, the recombinant complex (mMDH−CS) was less affected by increases in viscosity, indicating improved mass transport.

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In a nonchanneling system, OAA escaping into the bulk phase would be consumed by AAT, thus reducing the production of citrate. The coupled production of citrate by the recombinant complex was significantly less affected by the presence of a competing enzyme (AAT) or a viscous reagent (glycerol). Taken together with the structural evidence, these results demonstrate that assembly of sequential enzymes is important for efficient substrate channeling. In this work, the ESPs of commercial and recombinant mMDH−CS complexes were calculated, and it was shown that an electrostatic channeling path bridging active sites forms at the surface of commercial as well as recombinant enzymes. However, the recombinant complex with its active sites closely facing each other provides a channeling advantage over the complex of commercial enzymes whose active sites are further apart and facing oppositely. Moreover, the R65A mutation prevents association and the formation of the electrostatic channel in the metabolon. Mutated recombinant complexes retained only 50% of citrate production in the presence of 5 U mL^{-1} AAT, demonstrating that intermediate transport in the mMDH−CS(R65A) is more prone to being interrupted by competing pathways.

Characterization of Electrostatic Channeling by Transient-Time Analysis. Transient time (τ) is used to describe the lifetime of intermediates in coupled catalysis. In unassembled systems, intermediate species will require more time to diffuse to the next active site, resulting in longer observed transient times before steady state activities are reached. Substrate channeling can reduce this effect.[43] Here, the ordered bi−bi enzymatic reactions were treated as pseudo first-order by saturating the cofactors in the system. The overall initial reaction rates were limited by the OAA transport, which is dependent on the diffusion coefficient (D_i) of intermediates and the diffusing length (l) between active sites. Assuming that diffusion coefficients of OAA were not altered, the transient time is related to the diffusion distance length between the mMDH and CS active sites.

The transient times of OAA were measured in the native tissue mitochondrial metabolon and metabolons formed by commercial, recombinant, and mutant mMDH and CS (Figure 3, Table 2). The simulated electrostatic channeling pathway (40 Å) for OAA was shorter in the mMDH−CS complex formed by recombinant enzymes, compared to commercial enzymes (73 Å). Consequently, the transient time of OAA in the recombinant complex was measured to be 30 ± 11 ms, which is comparable to the value of 40 ± 5 ms measured with crude lysate containing the native tissue mitochondrial TCA cycle metabolon. In comparison, the transient time of OAA in the complex of commercial enzymes was almost 1 order of magnitude higher, with a value of 290 ± 40 ms. In the case of the mutant mMDH−CS(R65A) complex, the resultant transient time was increased to 880 ± 60 ms, approaching 1 s. According to these results, the channeling of OAA was fastest in the recombinant complex followed by the native tissue mitochondrial complex, complex of commercial enzymes, and the mutant mMDH−CS(R65A) complex.

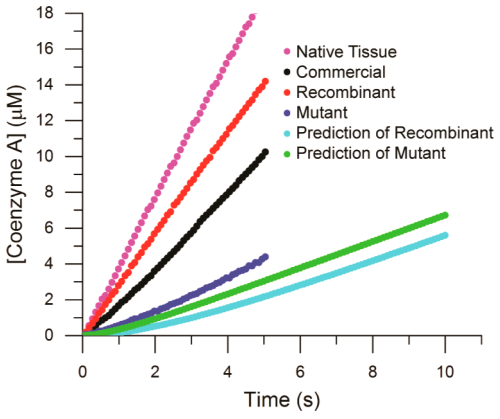

Figure 3. Characterization of OAA channeling *via* transient-time analysis where citrate production was measured over time. Predictions of recombinant and mutant complexes were determined using rate equations for each enzyme. Transient time for each enzyme sample was determined by extrapolating the linear line fitted to the curve to the time axis (Table 2).

Table 2. Measured and Predicted Transient Times of OAA[a]

sample	τ (ms)
native tissue	40 ± 5
commercial	290 ± 40
recombinant	30 ± 11
mutant	880 ± 60
prediction of recombinant	2500
prediction of mutant	900

[a]Transient times were determined from linear fits from Figure 3. Mean and standard deviation for experimental values were calculated from three independent experiments.

Agreeing with previous experiments, transient-time analysis also demonstrated that the mMDH−CS complex formed by recombinant enzymes in solution achieved similar channeling characteristics as the native tissue mitochondrial metabolon. The transient time of OAA in the recombinant mMDH−CS complex was reduced by 90% compared to the complex of commercial enzymes, indicating a higher channeling efficiency. The reduction of transient time in the recombinant complex, as compared to the complex of commercial enzymes, was larger than the expected value of 70%, according to the equation for one-dimensional random walk, $l^2 = 2D_i$.[44] This is likely a result of the better orientation of the active sites in the recombinant complex. In the complex of commercial enzymes, the active site clefts in mMDH are facing oppositely to CS and are open to the bulk phase, resulting in an increased chance of OAA escape. Although substrate channeling in both systems was found to be "leaky," it is evident that recombinant enzymes exhibit higher catalytic coupling efficiency.

An analytical approach has been developed which relates the substrate channeling phenomena to the Michaelis−Menten parameters of the enzymes.[37,43,45] A channeling probability parameter has been defined as $p_c p_r$, which can be obtained using the transient time, K_M, and V_{max} of the second enzyme in the complex. The applicable equation to the mMDH−CS system is defined as the following:[37]

$$\tau = \frac{K_{M,app,OAA}(1 - p_c p_r)}{V_{max,app}} \quad (1)$$

By using the ordered bi−bi rate equation and the kinetic parameters of the recombinant mMDH, CS, and CS(R65A) enzymes, simulated rates of citrate formation by the unassembled enzymes were calculated. From these, the predictions of transient times of OAA in recombinant mMDH−CS and mMDH−CS(R65A) systems were estimated

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to be 2.5 and 0.90 s, respectively (Table 2). These values represent the transient times with no interaction and no channeling, thus the probability parameter, $p_c p_r$, can be taken to be equal to 0. As shown by eq 1, $K_{M,app}/V_{max,app}$ is equal to the transient time in the case of no channeling. When this $K_{M,app}/V_{max,app}$ parameter is used together with the observed transient times of the complexes formed by the enzymes (0.03 and 0.88 s for the recombinant and mutant complex respectively), the combined channeling parameters are calculated to be 0.99 and 0.023 for the recombinant mMDH−CS and mMDH−CS-(R65A), respectively.

This analysis further confirms that the channel formation is disrupted in the mutant mMDH−CS(R65A) complex. The predicted and measured transient times are very similar for mMDH−CS(R65A) (0.90 s vs 0.88 s), which indicates that the intermediate OAA is channeling poorly after the arginine mutation. On the contrary, the measured time lag for the recombinant complex was 0.03 s, indicating efficient channeling of OAA within the metabolon. The probability parameter, $p_c p_r$, should approach 1 as intermediates are efficiently channeled,[37] and this value was found to be 0.99 and 0.023 for mMDH−CS and for mMDH−CS(R65A), respectively. A comparison of the $k_{cat}/K_{M,acetal\text{-}CoA}$ values for the recombinant wild-type (4.3 μM^{-1} s^{-1}) and mutant CS (5.6 μM^{-1} s^{-1}) as well as the $k_{cat}/K_{M,OAA}$ values (11 μM^{-1} s^{-1} for wild-type and 39 M^{-1} s^{-1} for the mutant CS) indicate that these results arise from changes in the transport efficiency of the complexes and are not due to major changes in kinetic activities.

Metabolic control analysis (MCA) provides a framework to understand how metabolic fluxes are regulated by enzymatic activities.[46,47] The TCA cycle is a highly regulated network in central metabolism. To further investigate the potential implications of the R65A mutation, the elasticity coefficients of CS and CS(R65A) with respect to substrate OAA were calculated. These parameters indicate how much the reaction rates are affected by changes in OAA concentrations and were estimated to be 0.79 for CS and 0.63 for CS(R65A) (eq S2) based on the steady-state substrate concentrations obtained with our model. Thus, the sensitivity of CS to OAA concentrations was decreased by the mutation. Taking the connectivity theorem into account, it can be concluded that this mutation would lead to an increased flux control coefficient, indicating a potentially increased role in regulating metabolic control. The potential impact on the flux control may explain why this mutation is rarely observed in nature.

Conclusion. Complex metabolic pathways, such as the TCA cycle, involve multiple enzymatic steps that require efficient mass transfer of intermediates between active sites. The metabolon formation within the TCA cycle and the interactions between malate dehydrogenase and citrate synthase in particular have been a major research focus. In this work, we presented the first direct evidence for metabolon formation among recombinantly produced mMDH and CS. The structural and kinetic analyses demonstrated that the recombinant versions of these enzymes self-assemble *in vitro*, similar to their native counterparts *in vivo*. Important residues for the enzyme interactions were identified, and site-directed mutational analysis was performed for the first time to investigate the substrate channeling among these enzymes. A single mutation in CS, R65A, along the positively charged patch connecting the active sites, disrupted the transport of the negatively charged intermediate, decreasing the overall channeling probability from 0.99 to 0.023. These results demonstrate the importance of substrate channeling in this critical biological pathway.

METHODS

Recombinant porcine mMDH and CS were constructed and expressed in *E. coli*. Highly purified recombinant enzymes and commercially purchased enzymes were chemically cross-linked with disuccinimidyl glutarate, and the trypsin-digested peptides were analyzed with liquid chromatography-tandem mass spectrometry (LC-MS/MS). Peptide cross-links were identified and complex structures were determined using previously published methods.[27] Mutations in CS were made by site directed mutagenesis. Kinetic parameters for an ordered bi−bi mechanism were determined spectrophotometrically for recombinant mMDH, CS, and CS(R65A). mMDH−CS complexes were analyzed for substrate channeling with coupled enzymatic assays. Detailed materials and methods are given in the Supporting Information.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.6b00523.

Supplemental Figures, Tables, and Materials and Methods (PDF)

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Author Contributions

[‖]These authors contributed equally to this work. B.B., K.E.G., and F.W. conducted the experiments. S.D.M. and S.B directed the research. B.B. and F.W. wrote the manuscript and all authors commented on the manuscript.

Notes

The authors declare no competing financial interest.

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