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Interaction between Citrate Synthase and Malate Dehydrogenase

SUBSTRATE CHANNELING OF OXALOACETATE*

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The interactions between pig heart citrate synthase and mitochondrial malate dehydrogenase or cytosolic malate dehydrogenase were studied using the frontal analysis method of gel filtration and by precipitation in polyethylene glycol. This method showed that an interaction between citrate synthase and mitochondrial malate dehydrogenase occurred but no interaction between citrate synthase and cytosolic malate dehydrogenase. Channeling of oxaloacetate in the malate dehydrogenase and citrate synthase-coupled systems was tested using polyethylene glycol precipitates of citrate synthase and mitochondrial malate dehydrogenase, and citrate synthase and cytosolic malate dehydrogenase. The effectiveness of large amounts of aspartate aminotransferase and oxaloacetate decarboxylase, as competing enzymes for the intermediate oxaloacetate, was examined. Aspartate aminotransferase and oxaloacetate decarboxylase were less effective competitors for oxaloacetate when precipitated citrate synthase and mitochondrial malate dehydrogenase in polyethylene glycol was used at low ionic strength compared with free enzymes in the absence of polyethylene glycol or with a co-precipitate of citrate synthase and cytosolic malate dehydrogenase. Substrate channeling of oxaloacetate with citrate synthase-mitochondrial malate dehydrogenase precipitate was inefficient at high ionic strength. These effects could be explained through electrostatic interactions of mitochondrial but not cytosolic malate dehydrogenase with citrate synthase.

In the last twenty years it has been demonstrated that sequential enzymes which operate within a metabolic pathway interact with each other to form highly organized complexes (1, 2). The term metabolon was introduced to describe such enzyme-enzyme complexes (3, 4). Among the mitochondrial enzymes of the Krebs tricarboxylic acid cycle, interactions occur between six of eight sequential enzymes: fumarase with mitochondrial malate dehydrogenase (mMDH)[1] (5), mMDH with citrate synthase (CS) (6–11), succinyl-CoA synthetase with α-ketoglutarate dehydrogenase complex (12), mitochondrial aconitase (mACO) with CS (13), NAD-dependent isocitrate dehydrogenase with mACO (14), and NAD-dependent isocitrate dehydrogenase with α-ketoglutarate dehydrogenase complex (15). Moreover, it has been shown that all of these enzymes bind to the inner surface of the mitochondrial inner membrane, whereas purified isozymes from other cellular compartments do not possess such binding abilities (13). Organized enzyme complexes may have several kinetic advantages for the cell (16, 17). One possible advantage is preventing intermediates from escaping into solution where they may be sequestered by other enzymes for use in different metabolic pathways. In other words, the close proximity of consecutive enzymes of a metabolic pathway may be used to increase the metabolic flow through this pathway, especially by channeling the substrate at reduced bulk substrate concentration.

However, there are methodological problems in demonstrating the *in vitro* existence of such intermediate channeling within enzyme-enzyme complexes. First, many of these interactions between enzymes are relatively weak, and the isolation of their complexes from cells is difficult because during the isolation procedure dilution effects or changes in the ionic strength will tend to dissociate the complexes. Different strategies have been used to obtain the active sites of consecutive enzymes in close proximity and to assess possible kinetic advantages of such an arrangement. Datta *et al.* (10) showed channeling of oxaloacetate (OAA) between mMDH and CS in a polyethylene glycol (PEG) precipitate of the two enzymes by using aspartate aminotransferase (AAT) as a trap for the intermediate OAA. Their results indicate that OAA is channeled between mMDH and CS, because large amounts of AAT are unable to trap OAA in the precipitate of the CS and mMDH. Another approach is the use of genetic fusions of consecutive enzymes, which may indicate the channeling of an intermediate substrate in solution. For instance, recent experimental work of Lindbladh *et al.* (11) on yeast mitochondrial CS (CS1) and yeast mitochondrial MDH (Mdh1p) connected by a short linker sequence into a fusion protein showed the existence of channeling of OAA between these two enzymes using the same competitive assay procedure as Datta *et al.* (10). Because of the short length of the linker connecting the CS and MDH enzymes, the orientational possibilities for the enzymes in the fusion protein are reduced, and a molecular model of the complex (11) was proposed using the known structure of the porcine enzymes. Elcock and McCammon (18) performed Brownian dynamics simulations to investigate the channeling of OAA in this model and provided evidence for an efficient electrostatically based channeling mechanism of OAA, even though mMDH and CS active sites are separated by nearly 60 Å in the model of the fusion protein. Another support for electrostatic channeling between two enzyme sites is provided by recent experimental work which shows that the efficiency of substrate

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[1] The abbreviations used are: mMDH, mitochondrial malate dehydrogenase; CS, citrate synthase; mACO, mitochondrial aconitase; OAA, oxaloacetate; PEG, polyethylene glycol; AAT, aspartate aminotransferase; CS1, yeast mitochondrial CS; Mdh1p, yeast mitochondrial MDH; OAADC, OAA decarboxylase; cMDH, cytosolic MDH; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

transfer within dihydrofolate reductase-thymidylate synthase is dependent on the ionic strength (19).

The same enzyme modeling strategy was extended with mACO, and recent computer graphic modeling studies provide a putative complex of porcine mACO·CS·mMDH complex (20). The model shows an electrostatically favorable channel between CS and mACO as well as the already reported mMDH-CS channel.

Using genetically prepared fusions with a short linker may not provide correct data about electrostatic channeling because the fusion of CS and mMDH may produce an unphysiological orientation of active sites in these constructs. Elcock *et al.* (21) recently developed a kinetic model for a coupled two-enzyme system containing a third enzyme that competes for the common intermediate. They then used the kinetic data of Lindbladh *et al.* (11) for CS1 and Mdh1p and showed for the free coupled enzyme system using free mMDH and CS, in the presence of increasing amounts of the competing enzyme AAT, that the theoretically derived curve matches exactly the experimentally observed data. When the kinetic data for CS1/Mdh1p fusion protein in the presence of AAT are analyzed with their kinetic model, the experimental data fit a theoretical curve that indicates 74% channeling of the OAA in the fusion protein.

In this study, we extend the previous work of Datta *et al.* (10) to a study of pig heart CS and mMDH in PEG by using the frontal analysis variant of the Hummel-Dreyer (22) method of gel filtration and by studying the kinetics of co-precipitates of these enzymes in PEG solution. Because there is a great difference between conditions generally used to characterize biochemical reactions *in vitro* and conditions that actually exist in the living cells, the use of crowding agents such as PEG make it possible to induce the formation of enzyme complexes *in vitro* with conditions which reproduce the crowding effect in cells. To ensure that the use of AAT as an OAA trap does not give special results, we also used a second OAA trapping enzyme, OAA decarboxylase (OAADC). By means of these two different OAA trapping reactions, the substrate channeling of OAA within the PEG-precipitated CS and mMDH was demonstrated. No substrate channeling of OAA within a PEG precipitate of CS and cytosolic MDH (cMDH) was detected using the trapping method. We also report the effect of ionic strength on the PEG precipitation and on the OAA channeling of the enzymes.

EXPERIMENTAL PROCEDURES

The following enzymes were used: pig heart mMDH and pig heart cMDH (EC 1.1.1.37), pig heart CS (EC 4.1.3.7), AAT (EC 2.6.1.1), and OAADC (EC 4.1.1.3) were obtained from Sigma. Malate, NADH, NAD, OAA, DTNB, and glutamate were also from Sigma. PEG (M_r = 4000) was purchased from Fluka (Switzerland). Superdex 200 was obtained from Pharmacia LKB (Sollentuna, Sweden). All other chemicals used were of the highest purity available.

Enzyme Preparation—Salt and buffer exchanges from the commercial enzyme suspensions were made by using Sephadex G-10 columns just prior to the experiments.

Enzyme Assays—CS activity was determined using 0.2 mM acetyl-CoA, 0.5 mM OAA, and 0.1 mM DTNB in 100 mM Tris-HCl (pH 7.5) (23). The reaction was followed spectrophotometrically at 412 nm. MDH was assayed using 0.1 mM NADH and 0.1 mM OAA in 50 mM Tris-HCl (pH 7.5) at 340 nm (24). AAT was assayed with 30 mM aspartate, 2.5 mM α-ketoglutarate, 0.05 mM pyridoxal phosphate, 0.4 mM DTNB, 0.1 mM acetyl-CoA, and excess pig CS in 100 mM potassium phosphate buffer (pH 7.5) at 412 nm (25). OAADC was assayed with 40 mM triethanolamine, 9.5 mM Tris-HCl (pH 8.0), 0.5 mM $MnCl_2$, 0.3 mM NADH, 2.3 mM OAA, 9.2 units/ml lactate dehydrogenase (26). Extinction coefficients were assumed to be 13,600 and 6300 M^{-1} cm^{-1} for DTNB and NADH, respectively. One unit of enzyme liberates 1 μmol of product/min. All reactions were measured at room temperature.

Coupled Enzyme Reactions—The overall reaction of malate to citrate catalyzed by MDH and CS, either free or as a PEG CS-MDH precipitate, was monitored in 10 mM malate, 4 mM NAD, and 0.1 mM acetyl-CoA using 0.4 mM DTNB at 412 nm in 10 mM potassium phosphate buffer (pH 8.1). Either AAT (10 units) or OAADC (10 units) were used as a competing enzyme for OAA. For AAT, 10 mM glutamate was added to the reaction mixture. For OAADC, the assay was performed in a Tris-HCl buffer with 1 mM $MnCl_2$. Reactions were measured at 10 °C. Enzyme concentrations are indicated in the text.

In 30% PEG, the rate of the coupled reaction was one-fourth that in the absence of PEG. The rate of CS in 30% PEG was similarly decreased. This is in agreement with the report by Datta *et al.* (10). The rates of AAT and OAADC were not affected by 30% PEG.

Frontal Analysis Chromatography—The gel filtration column was a Superdex 200 of 0.7 × 30 cm pre-equilibrated in 10 mM potassium phosphate buffer (pH 8.0) with 10% PEG. The optical density of the eluted solution was monitored at 280 nm with a Pharmacia LKB optical unit UV-1. The enzymes, mMDH and cMDH, were added to the column individually to establish their elution volume. The column was then equilibrated by the same buffer containing CS (0.2 mg/ml). Solutions of mMDH and cMDH were injected into the pre-equilibrated column to determine whether a stable complex was formed. The column was thermostatted at 4 °C, and the flow rate was 0.1 ml/min. Fractions (0.5 ml) were collected, and the activities of CS and MDH were determined as described above.

PEG Precipitation—MDH-CS complexes were prepared by incubating the two enzymes at 2 mg/ml in 10 mM potassium phosphate buffer (pH 8.0) containing 10 or 25% of PEG (w/v) for mMDH and cMDH, respectively, at 10 °C for approximately 1 h. After incubation, the suspension was centrifuged at 10,000 × *g* for 20 min at 4 °C, giving a pellet which was then homogeneously suspended in 30% PEG.

RESULTS

A frontal analysis method of gel filtration in PEG was used for detecting enzyme-enzyme interaction between CS and the isoenzymes of MDH. The column of Superdex 200 was equilibrated with 10 mM potassium phosphate buffer (pH 8.0) and 10% PEG. mMDH then was added to the column. As shown in Fig. 1*A*, the frontal elution volume of mMDH was about 15 ml. The column was then equilibrated with the same buffer containing 0.2 mg/ml of CS. When a mix of 0.2 mg/ml of CS and 0.1 mg/ml of mMDH was applied to the column, the frontal elution volume of mMDH was decreased to 5 ml (Fig. 1*A*), which indicated complex formation between CS and mMDH. Under the conditions used for this analysis, no precipitate was formed in the fractions of CS and mMDH that eluted early from these columns. The same experiment was performed with the same amount of cMDH (Fig. 1*B*). The presence of CS did not change the elution volume of cMDH. Therefore, the decrease of the frontal elution volume of mMDH in the presence of CS indicated a specific interaction between mMDH and CS. The column was calibrated with four marker proteins (thyroglobulin, apoferritin, CS, and myoglobin) in the presence of 10% PEG (Fig. 1*C*). *Arrows* show the elution volumes of the same proteins in 100 mM potassium phosphate buffer without PEG.

Next, the dissociation of the PEG CS-MDH complexes prepared as described under "Experimental Procedures" was investigated. Samples of the precipitated CS-MDH complexes (protein concentrations, 2 μg/ml) were incubated in a medium with the different PEG concentrations (0 or 30%) and ionic strengths (10 or 200 mM KPO_4) for 2 min, and the dissociation of CS-MDH complexes was followed by the decrease in turbidity at 650 nm (Fig. 2). It should be noted that the CS-MDH complexes very quickly dissociate when incubated without PEG (the absorbance at 650 nm rapidly decreased). However, when the suspension of the solid state enzymes was incubated in 30% PEG at low ionic strength, no decrease in turbidity was detected. Increasing the ionic strength at 30% PEG caused, over a period of time, the dissociation of CS-mMDH (data not shown). During the first 2 min (time required for kinetic measurements), about 90% of the CS-mMDH precipitate remained in the solid state at high ionic strength (Fig. 2*A*). The same experiments were performed with CS-cMDH complex (Fig. 2*B*). These results demonstrated that solid state complexes of CS-mMDH and CS-cMDH were stable at 30% PEG even at high

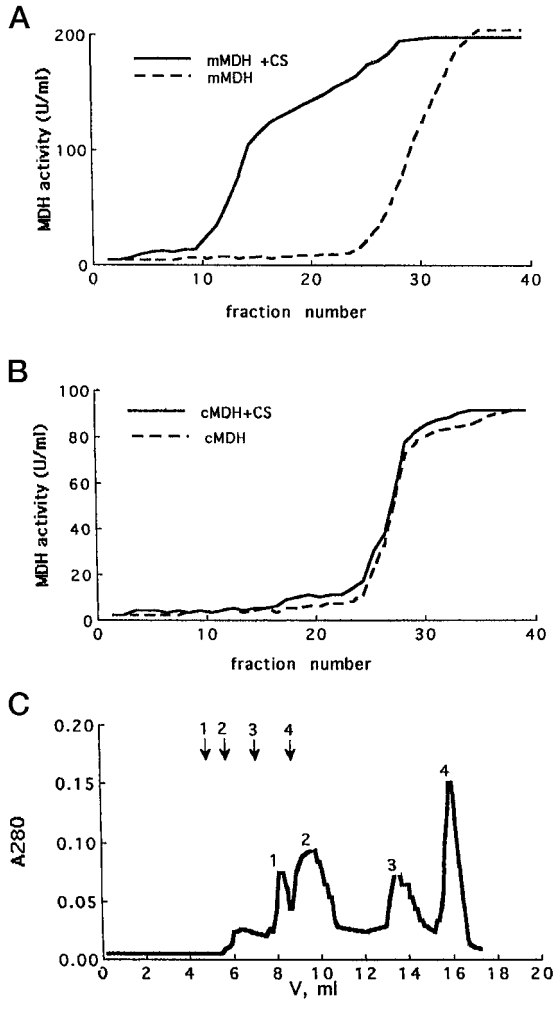

FIG. 1. **Influence of CS on elution profiles of mMDH and calibration of this column (*A*) and cMDH (*B*) on Superdex 200 in presence of 10% PEG (*C*).** 1-thyroglobulin (669 kDa), 2-apoferritin (443 kDa), 3-CS (100 kDa), 4-myoglobin (17.6 kDa). *Arrows* show the elution volumes of the same proteins on this column without PEG.

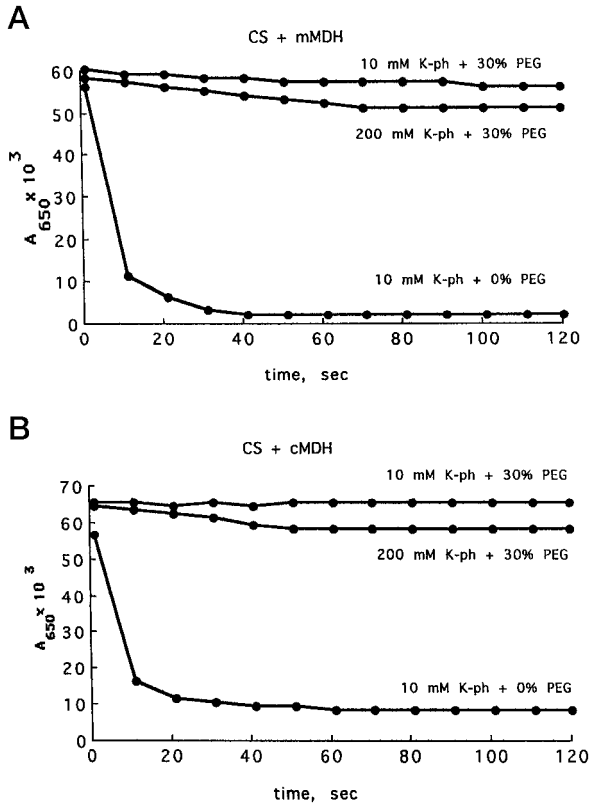

FIG. 2. **The effect of PEG and ionic strength on dissociation of complexes between mMDH (*A*) and cMDH (*B*) and CS.** Complexes of enzymes (2 μg/ml of solution) in PEG were incubated at different concentrations of PEG and potassium phosphate buffer for 2 min.

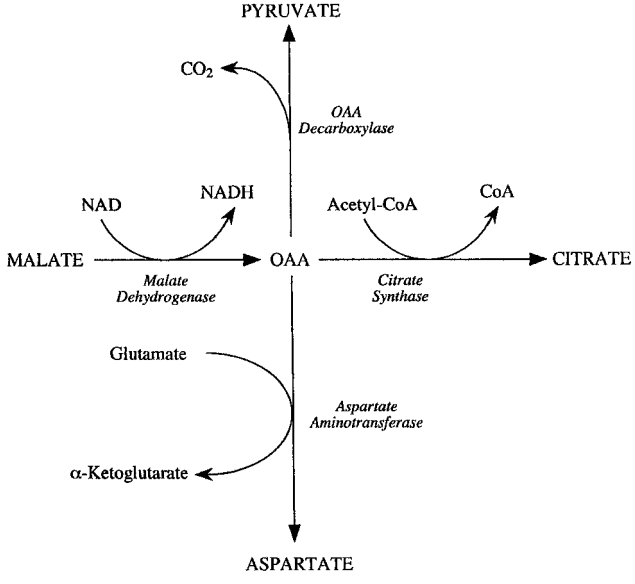

FIG. 3. **Reaction scheme for AAT and OAADC competition for OAA formed in the coupled MDH-CS reaction.**

ionic strengths. This made it possible to study the kinetic properties of these complexes.

To determine whether there was a kinetic advantage of the coupled systems enzyme associated in PEG compared with free enzymes, two different OAA-trapping enzymes were used for the coupled CS-MDH enzyme reaction. In the coupled systems, the rate of conversion of malate to citrate *via* OAA catalyzed by the enzymes MDH and CS was tested in the presence of either excess AAT or excess OAADC. The same ratio of enzymes was used for all experiments: 1:2:50 by activity for CS-MDH-AAT or OAADC. A scheme of the coupled enzyme reaction with the OAA traps is shown in Fig. 3.

This coupled system was used as a test for channeling of the intermediate OAA between the enzymes CS and MDH. First, AAT was added to the reaction mixture CS-mMDH or CS-cMDH. As seen in Fig. 4, PEG concentration and the ionic strength of the reaction mixture caused different kinetic behavior of the coupled systems of CS-mMDH and CS-cMDH. A reaction mixture without PEG (*i.e.* no complex formed), the coupled reaction (*i.e.* the production of CoASH), was reduced to 28% by AAT in the CS-mMDH mixture and reduced to 26% for the CS-cMDH mixture, which showed good trapping of the OAA by AAT at low ionic strength. In 30% PEG in which a precipitate for CS-cMDH as well as CS-mMDH exists, the coupled reaction was reduced only to 66% by AAT in CS-mMDH, indicating channeling of OAA from mMDH to CS. However, the coupled reaction rate was still reduced to 27% by AAT for the CS-cMDH precipitate which indicated no channeling of OAA. With 30% PEG, an increase in ionic strength to 200 mM KPO_4 resulted in a 3-fold decrease in the coupled reaction by AAT in the CS-mMDH complex compared with that in 10 mM KPO_4, similar to the result with free enzymes. However, with CS-cMDH, the higher ionic strength did not significantly influence the coupled reaction since AAT was already effec-

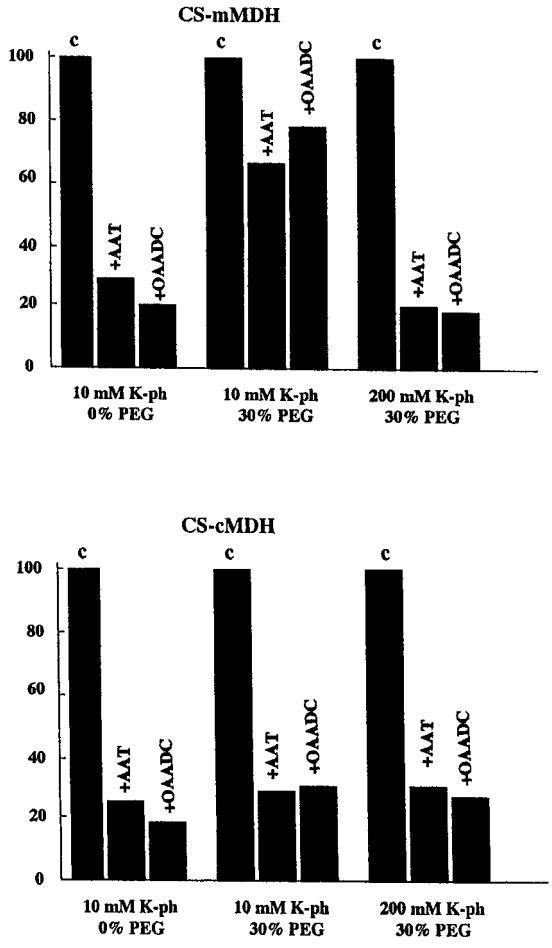

FIG. 4. **The effect of AAT and OAADC on coupled reactions catalyzed by mMDH and cMDH and CS.** The same ratio of enzymes was used for all experiments: 1:2:50 by activity for CS-MDH-transaminase or OAADC. Absolute concentrations were 0.2 unit of CS, 0.4 unit of mMDH or cMDH, and 10 units of AAT or OAADC per ml of reaction mixture. The initial rate of the coupled reaction in 30% PEG was 4-fold less when compared with the activities in the reaction mixture without PEG. *c*, control.

tively trapping the OAA that was not being channeled. Similar results were obtained when OAADC was used as the OAA-trapping enzyme instead of AAT. Depending on the MDH isoenzyme used and the ionic strength of the reaction mixture, different effects of the addition of trapping enzymes were observed on the kinetics of coupled systems with CS-mMDH and CS-cMDH. At low ionic strength and in the presence of 30% PEG in the reaction mixture, a significant difference in the coupled reaction was observed depending on the MDH isoenzyme used. In the case of CS-mMDH complex, the coupled reaction was reduced only to 78% of the control but was reduced to 28% of the control in the case of CS-cMDH. An increase in the ionic strength to 200 mM resulted in a dramatic decrease of channeling in the coupled reaction in the presence of OAADC in the case of CS-mMDH, whereas no significant effect was observed of an increase in ionic strength with the CS-cMDH complex.

DISCUSSION

There is a difference between conditions generally used to characterize biochemical reactions *in vitro* and conditions that actually exist in living systems. Typically, biochemical reactions are studied *in vitro* in dilute buffer solutions. However, the interior of a living system differs from these conditions. For example, as shown for mitochondria, matrix conditions are crowded with many macromolecules that are bound and organized in multienzyme complexes. Crowding conditions result in the enhancement of macromolecular associations and hindrance of macromolecular diffusion (27–29). There are speculations that diffusion of small molecules is affected by less than an order of magnitude by macromolecular crowding, and therefore metabolism proceeds predominantly via the diffusion of small molecular metabolites between the various active sites of enzymes catalyzing sequential reactions (27–29). The use of crowding agents, such as PEG, reduces the solubility of proteins (30–32) which also induces the formation of enzyme-enzyme complexes, approximating those conditions *in situ*. The present experiments confirmed and extended evidence for the interaction between CS and mMDH in PEG. The frontal analysis method of gel filtration showed that the elution volume of mMDH was decreased when the column was equilibrated with CS (Fig. 1). When mMDH was replaced with cMDH in these experiments, the elution volume did not change, showing the specificity of the interaction between the CS and mMDH in PEG solutions. It should be noted that the elution volumes of both mMDH and cMDH without CS in PEG were larger than the bed volume of the column (11.6 ml). The four marker proteins in the presence of PEG also had larger elution volumes than without PEG. Larger elution volumes of proteins in gel filtration in the presence of PEG than in its absence may be explained by interactions between PEG and both the proteins and the gel matrix (33).

The interaction of CS and mMDH to form an enzyme-enzyme complex in PEG solutions was found by Halper & Srere (7), who showed that the solutions of CS and mMDH in 14% PEG result in a precipitation of the two enzymes, in contrast to solutions with other enzymes or to a mixture with CS and cMDH.

The results reported here demonstrated that the enzyme-enzyme complexes between CS and mMDH obtained by the precipitation in PEG did not dissociate at high PEG concentrations, even for low concentrations of CS and mMDH. An increase in ionic strength caused the dissociation of CS-mMDH complexes, but the complexes (about 90%) remained in a solid state for at least 2 min (the time required for the measuring of enzyme kinetics of coupled reactions). This allowed a study of the kinetics of these complexes.

Datta *et al.* (10) demonstrated that the coupled reaction catalyzed by the solid state complex of CS-mMDH is less sensitive to the AAT trapping of OAA than the free enzyme coupled reaction, which indicates channeling of this intermediate by the solid state complex. Thus, quantities of AAT that reduce the citrate production reaction rate with free mMDH and CS by 90% do not significantly affect rates with comparable amounts of the mMDH-CS complex. The current results agreed with the previous report. In addition, this study showed protection of the OAA intermediate of the CS-mMDH complex using OAADC as an additional trapping enzyme for OAA. These results suggested that the channeling of OAA in the precipitate of CS-mMDH protected it from the trapping by large excesses of AAT and OAADC. We also show that the CS-cMDH precipitate was unaffected by AAT and OAADC, in that the trapping was the same as that of free enzymes. The conclusion is that the kinetic advantages of CS-mMDH complexes were because of direct channeling of substrate OAA from mMDH to CS and not because of the enzymes being in a solid state precipitate and restricting the diffusion of OAA.

Recent theoretical (18) work suggests that the substrate channeling of OAA is because of the electrostatic nature of the surface of the proteins between the two active sites, and the efficiency of substrate transfer is enhanced by electrostatic effects. The results reported here on ionic strength effects pro-

vide supporting evidence for the theoretical simulations that substrate channeling in CS-mMDH is because of electrostatic mechanisms.

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