Substrate Channeling of Oxalacetate in Solid-state Complexes of Malate Dehydrogenase and Citrate Synthase*

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Current evidence suggests that mitochondrial matrix enzymes exist in solid-state, multienzyme complexes in vivo. Addition of polyethylene glycol to a solution containing malate dehydrogenase and citrate synthase generates such a solid-state, enzyme complex in vitro at enzyme concentrations permitting kinetic measurements. Suspensions of the isolated, solid-state, heterologous complexes of these enzymes were used to study the coupled reactions of citrate synthesis from malate, NAD, and CoA. The particles appear to be about 1 μm in diameter. Considering the ratio of enzyme to oxalacetate molecules in or at the surface of the solid-state particles, one would expect oxalacetate to be converted to citrate within a few molecular distances of the site of oxalacetate generation. This model of “substrate channeling” (or alternatively a direct transfer of oxalacetate between enzymes) is supported by experiments with excess aspartate aminotransferase and glutamate dehydrogenase added to the solution phase to give a reaction competing with the synthase for bulk phase oxalacetate. Quantities of aminotransferase that reduce the citrate reaction rate with soluble dehydrogenase and synthase by 90% do not significantly affect rates with comparable amounts of the dehydrogenase-synthase complex. We suggest that similar substrate channeling can occur in vivo and discuss the possible advantages provided thereby.

Current evidence indicates that many of the enzymes of the mitochondrial matrix can form specific complexes with metabolically adjacent enzymes or inner mitochondrial membrane components. Examples include binary complexes among the enzymes, glutamate dehydrogenase (1), aspartate aminotransferase (2), malate dehydrogenase, and citrate synthase (3) and complexes between pyruvate dehydrogenase complex and citrate synthase (4), α-ketoglutarate dehydrogenase complex and succinate thiokinase (5), citrate synthase and thiolase (6), and carbamyl-phosphate synthetase and ornithine transcarbamylase (7). Ternary enzyme complexes between aspartate aminotransferase, glutamate dehydrogenase, and carbamyl-phosphate synthetase (1) and between fumarase, malate dehydrogenase, and either aspartate aminotransferase or citrate synthetase (8) have also been reported. The binding of several of the soluble dehydrogenases to complex I (9), binding of fatty acid β-oxidation enzymes to inner mitochondrial membranes (10), and the chemical cross-linking in situ of enzymes from the mitochondrion (11) have also been reported. The extremely high protein and low water content of the mitochondrial matrix also suggest that the enzymes exist in solid-state aggregates rather than in a solution phase (12). An extensive review of the organization of proteins in mitochondrial matrix has been recently written (13).

These reports have promoted the idea that these enzyme complexes can permit partial or complete channeling of the dissociable intermediates between the enzymes, i.e. the product of the first enzyme is utilized by the second enzyme before the intermediate diffuses from the molecular complex into the surrounding bulk phase. Such substrate channeling could provide significantly different catalytic properties than possible by homogeneous phase catalysis. Substrate channeling has been demonstrated in several systems. The direct reaction of the nondissociable pyridoxamine of aspartate aminotransferase with glutamate dehydrogenase and NADP (14), although not substrate channeling as defined above, is a similar reaction between two metabolically adjacent enzymes. Substrate channeling, however, has not been demonstrated to our knowledge for the above referenced complexes of mitochondrial matrix enzymes. This is largely due to the fact that either the extent of enzyme association is small in the solutions studied or that the complexes are solid-state precipitates with which it is difficult to do kinetic studies.

For reasons discussed below, substrate channeling seems especially likely if the enzyme complex exists as a solid-state aggregate. In this paper, we report studies of substrate channeling in the solid-state malate dehydrogenase-citrate synthase complex induced by polyethylene glycol (PEG) (3). Our past studies (15) indicate that the enzyme complex is highly insoluble, and thus little association occurs in the solution phase. Thus, kinetic properties of the solution are overwhelmingly those of the unassociated enzymes, which prevents a kinetic characterization of the complex in the solution phase. Moreover, the solid-state complex may be more biologically relevant (12). Previous, elegant studies of microenvironmental and enzyme-enzyme proximity effects with these coupled reactions have used co-immobilized and cross-linked enzymes (16, 17). However, cross-linked enzymes may force the two molecules into an unfavorable and unphysiological orientation of active sites. Our present study also differs in the use of local enzyme concentrations nearly 5 orders of magnitude higher, which should be more physiological and an important factor for efficient substrate channeling. A more direct and sensitive measure of substrate channeling than that based on lag transients is also used in the present study.

EXPERIMENTAL PROCEDURES

Materials—Ammonium sulfate suspensions of the porcine heart enzyme (citrate synthase; EC 4.1.3.7), mitochondrial malate dehydro-

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*This work was supported by Grant GM16916 from National Institutes of Health. This is a Journal Article 54750 of the Oklahoma Agricultural Experiment Station. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: PEG, polyethylene glycol; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid).
Substrate Channeling of Oxalacetate

Strategies and Results—To ensure that the kinetic measurements would characterize the solid-state enzymes (malate dehydrogenase, citrate synthase, or their heterocomplex), we sought conditions with which less than 10% of the total enzyme activities were contributed by solution-phase enzymes. Even though PEG lowers the solubilities of the individual or complexed enzymes greatly, the resulting total enzyme concentrations at solubility equilibria were too high for kinetic measurements; attempts with both continuous or stopped-flow methods failed due to the extremely high viscosities of the PEG solutions. Therefore, our procedure (see “Experimental Procedures” for details) was to transfer a small reaction cuvette and make kinetic measurements before the enzyme suspension dissolved significantly. Filtration and centrifugation methods were tried to quantitate the amount of solid-state enzyme remaining after the reaction period, but were found too slow and unreliable. Therefore, differences between the activities of solution- and solid-state enzymes were used to indicate that the enzymes remained in the solid state during the kinetic measurements.

Fig. 1 illustrates the differences in apparent specific activities of the dehydrogenase between the solution and solid states over a range of PEG concentrations. The reaction was initiated by addition of the solid-state enzymes (lower curve), or the enzymes were incubated for 5 min in the cuvette before starting the reaction (upper curve). Similar results were obtained when the synthase activity was measured. High PEG concentrations are needed to slow rates of dissipation of the solid-state enzymes, yet too high a PEG concentration would have resulted in the first 15–20 s to minimize solubilization of solid-state enzymes. The reactions were started by adding 10–50 µl of enzyme or substrate solution to the propeller and stirring for 15–20 s. Adequate mixing was judged by the fact that reaction rates observed after 10 s of stirring were the same as for longer stirring periods. Reactions were started by addition of enzyme, or when enzymes were preincubated, by addition of oxalacetate. For experiments with preincubated enzymes, the solution was stirred for 10–15 s upon addition of the enzymes and again for 10–15 s half-way through the incubation time.

RESULTS AND DISCUSSION

Malate dehydrogenase-citrate synthase solid-state aggregate preparations were made by recombining the two enzymes at 2 mg/ml in 10 mM potassium phosphate buffer at pH 7.5. PEG was added soon after preparing these solutions, since the enzymes are unstable in low ionic strength buffers in the absence of PEG. High ionic strength buffers are undesirable for preparing and keeping solid-state aggregates of the dehydrogenase and synthase. On the other hand, dissolution of the solid-state aggregates is slow enough to tolerate higher ionic strengths during the short period of the kinetic measurements. Therefore, high ionic strength buffers were preferred for all kinetic measurements to provide better pH control and enzyme stability. Protein concentrations were determined by measuring absorbance at 280 nm using specific absorbivities of 1.78, 0.250, and 1.40 (mg/ml)^{-1} cm^{-1} for the synthase (19), dehydrogenase (20), and transferase (21), respectively. All enzymes were spectrophotometrically assayed in a 1-cm path length cuvette. Assays for determining quantities of each enzyme were done at room temperature with an assay buffer of 100 mM potassium phosphate, pH 7.5; other kinetic measurements were done at lower temperatures (10 °C) to reduce the solubilization of the solid-state enzymes. Citrate synthase was assayed (22) using a suspension of 0.1 mM oxalacetate, 0.4 mM DTNB and measuring the absorbance at 412 nm. CoASAc was made by acetylation of CoA with acetic anhydride (23). Malate dehydrogenase was assayed using 0.2 mM NADH, 0.05 mM oxalacetate and 340 nm light. Aminotransferase was assayed (24) with 0.5 mM oxalacetate, 20 mM glutamate and 280 nm light. Absorptivities assumed were 13,800, 6,200, and 528 (mg/ml)^{-1} cm^{-1} for the DTNB, NADH, and oxalacetate (24) reactions, respectively. Specific activities of enzymes were 120, 500, and 100 units/mg for the synthase, dehydrogenase, and transferase, respectively, where unit is 1 unit of activity in micromoles of product/minute.

Malate dehydrogenase-citrate synthase solid-state aggregates were prepared by recombining the two enzymes at 2 mg/ml in 10 mM potassium phosphate buffer containing 10% PEG (w/v) at 10 °C for approximately 1 h. Independent solubility measurements established that the individual enzymes were soluble with these conditions; thus, only heteroenzyme precipitation is expected. The turbid suspension was centrifuged at 10,000 × g for 40 min at 10 °C giving a pellet, which was homogeneously suspended in 30% PEG. Calculations from solubility data indicate that no more than 0.3% of either enzyme exists in the solution phase of this suspension. The aggregate, thus formed, was stored at 0–4 °C. The enzymes were stable for at least 3 weeks in the presence of 30% PEG. Enzyme quantities in the aggregates were determined by enzyme activity measurements on aliquots of dissolved suspensions.

Coupled Enzyme Reactions—Reactions were measured at 10 °C, and the production of CoA was monitored with DTNB at 412 nm. Measurements with varying DTNB established that the DTNB reaction did not cause a significant lag response or rate limitation (both effects occur at lower temperatures and higher enzyme concentrations). Nitrogen was passed through the cell holder to prevent fogging of optical windows. The reaction mixture contained 40 mM malate, 4 mM NAD, 0.1 mM CoASAc, 4 mM glutamate, 0.4 mM DTNB, 34% PEG, 100 mM potassium phosphate buffer, pH 7.5, and enzyme concentration as specified in the text. Concentrated stock solutions of substrates were made in 100 mM potassium phosphate buffer and the pH was adjusted to 7.5 with KOH when necessary. To circumvent the problem of solubilization of the enzyme aggregates, yet achieve a thorough mixing with the highly viscous PEG solution, an efficient stirring apparatus was constructed. This used a small (1 × 1 inch, diameter) Plexiglas® rod (4 × 0.5 cm) twisted into a helix to enhance vertical flow in the cuvette at gentle speeds to avoid entrapping air at the solution surface. A simple control circuit was constructed to maintain the motor speed independent of the load, but continuously adjustable by the user. The motor could be fixed to the cuvette holder or held by hand for stirring. Because the stirring rod was translucent (absorbance of about 0.5) and the response time of the recorder was slower than the rotational speed of the propeller, kinetic measurements could be made while stirring, although in this study stirring was discontinued after the first 15–20 s to minimize solubilization of solid-state enzymes. The reactions were started by adding 10–50 µl of enzyme or substrate solution to the propeller and stirring for 15–20 s. Adequate mixing was judged by the fact that reaction rates observed after 10 s of stirring were the same as for longer stirring periods. Reactions were started by addition of enzyme, or when enzymes were preincubated, by addition of oxalacetate. For experiments with preincubated enzymes, the solution was stirred for 10–15 s upon addition of the enzymes and again for 10–15 s half-way through the incubation time.
abolish activity differences between solid and solution states of the enzymes. Thus, 34% PEG was considered near optimal and used for further study. Fig. 1B indicates that the enzymes remained in the solid state if the reaction was initiated with no preincubation, but dissolved within 5 min of preincubation.

As an aside, we point out that enzyme described by the upper curve of Fig. 1A appears to be completely dissolved. That is, the decreasing activities with increasing PEG concentrations seen in the upper curve are due to PEG inhibition of the soluble enzyme rather than precipitation of enzyme. Complete solubility of the enzymes after preincubation or when added as soluble enzymes is indicated by the following reasons. First, the specific activities of enzyme were independent of enzyme concentration (data not shown). Also, substrate channeling (discussed below) was essentially abolished if the solid-state complex was preincubated 10 min before starting the reaction. Furthermore, in our experience, when a soluble enzyme mixture is used for the stock solution, it takes several minutes before any evidence of precipitation develops (turbidity or light scattering data) unless higher PEG or protein concentrations are used. Therefore, the fact that specific activities from the enzyme complex after preincubation approached within experimental error of those obtained with soluble enzyme stock (Fig. 1B) also indicates solubilization of the enzyme complex. Subsequent conclusions concerning substrate channeling, however, do not require knowledge of how the activities in the lower curve of Fig. 1B increase with time.

To test for escape for oxalacetate from the solid-state enzymes into the solution phase, high concentrations of aspartate aminotransferase with glutamate were used to trap oxalacetate. Table I shows that the excess aminotransferase trapped virtually all of the dehydrogenase-generating oxalacetate in the solution phase. AAT, aspartate aminotransferase.

**Mechanisms of Substrate Channeling**—Two types of molecular mechanisms for substrate channeling should be considered. In one, the intermediate is passed to the second enzyme before dissociation from the first enzyme. This mechanism has been demonstrated for a number of soluble enzymes, the recent studies of Bernhard and co-workers being especially interesting (27). Substrate channeling, however, can also be obtained when the intermediate dissociates from the first enzyme, if the local concentration of the second enzyme is sufficient to utilize the intermediate before it diffuses away.

We consider our results from this perspective next. Dark-field microscopic observations on an aliquot from a suspension of the solid-state enzyme complex indicate particle sizes of about 1-μm diameter. Assuming partial specific volumes of protein within the solid state of 0.75 ml/g, a 1-μm particle would contain about 10 million protein molecules. From enzyme activity measurements on aliquots of dissolved solid-state enzyme complexes, we observed a ratio of 1.98 ± 0.45 (w/w) synthase/dehydrogenase (10 measurements). This corresponds closely to a 3:2 molar ratio of synthase/dehydrogenase. From this, it follows that the density of citrate synthase is about 8 mM in the solid state. Considering that the reactions were still in the initial velocity stage, concentrations of free oxalacetate (unbound to protein) in the interstitial fluid of the solid-state aggregate were probably less than 10 μM. Thus, on the average, each oxalacetate molecule would be surrounded by about 1000 molecules of citrate synthase. Under these conditions, it is reasonable that most oxalacetate molecules would be converted to citrate before escaping from the solid-state aggregate. This same high ratio of enzyme to oxalacetate molecules would also exist if the enzymatic activity were confined to the surface of the solid-state particles.

It is more likely that enzymatic activities were confined to the surface of the particles as indicated by the following evidence. PEG would not be expected to be within the domain of the solid-state particles, since it acts to exclude these enzymes from its environment, as indicated by several experiments (3, 15, 28). Thus, molecules within and on the surface of the particles should be free of the extensive PEG inhibition (about 20-fold) found in the solution phase. The specific activities observed for both enzymes, therefore, are about 1-2% of those expected for uninhibited enzyme in the solution phase. A monomolecular shell of these enzymes at the surface of a 1-μm diameter particle would contain 1% of the total enzyme and therefore could account for the observed specific activities. If the reduced specific activities were due to slow diffusion of the substrates into the interior of the solid-state particles, some evidence of this diffusion barrier should have been observed in the various kinetic measurements. Yet, the same ratio of activities (solution to solid state) was observed with the dehydrogenase reaction whether the forward or reverse reaction was measured. Since the substrate concentrations are at least 80 times greater for malate oxidation, the same ratio would not be expected with highly diffusion controlled reactions. Also least-squares fits to full-progress curves of the synthase reaction within the solid-state complex gave a K_m of 3.7 μM with low standard deviation and a high quality fit (29). Initial velocity data gave a similar value, but with a greater uncertainty. These values are very close to those we found for the soluble enzyme in the same solution and close to literature values (30). Furthermore, molecular models of CoASac indicate that it is too large to diffuse through close packed spheres of the size of the enzymes used (12).

The kinetics of surface-bound enzymes catalyzing coupled reactions have been analyzed by Goldman and Katchalski (31). With high concentrations of the two surface-bound enzymes, the intermediate is converted to product with negligible loss of the intermediate to the bulk phase. This is the result of an unstarved water layer surrounding the solid-state...
surface, which slows the transport of the intermediate away from the surface. Is this compatible with our data on the individual reactions using exogenous oxalacetate, with which we see no diffusion effects? Our analysis of model equations incorporating the diffusion process demonstrates that the mechanism is compatible with our data if a sufficiently small unstirred water layer exists. Thus, our present data do not distinguish between these two types of substrate channeling mechanisms: direct transfer without dissociation or dissociation but local consumption.

Physiological Implications—In many cases, the weakness of the enzyme associations detected in vitro, especially at physiological ionic strengths, raises questions about their existence in vivo. Several facts and considerations reduce these doubts. It is likely that there are additional interactions in the more complex physiological environment which stabilize these associations. Some of these additional factors have been demonstrated in vitro recently (1, 32). The impressive specificity for enzyme partners in many cases also argues for a physiological function of the complexes. Furthermore, the extremely high protein, but low water content of the matrix (12), should greatly enhance enzyme associations in vivo over those detected in vitro. The excluded volume effect of the high protein concentration alone can provide tens of kilocalories/mole of energy to stabilize enzyme associations (30) that would be undetectable with typical conditions in vitro.

The use of PEG deserves comment. As mentioned above, this polymer appears to exclude enzyme molecules from its environment so that an unphysiological polymer-enzyme complex is not expected. Increasing evidence (28) supports the view that the polymer effects are primarily due to the excluded volume it provides, which, because it is a random coil, is much greater than an equal weight concentration of globular proteins. The effect of PEG on proteins is not due to altered activity of water. At 14% PEG, in which considerable enzyme complex is formed (3), the vapor pressure of the water is within 99% of that of pure water (34). Therefore, PEG may be considered an inert macromolecule providing large excluded volume effects as exist in the mitochondrial matrix, which contains about 560 mg of protein/ml (12, 13).

From the content of malate dehydrogenase and citrate synthase (35) and mitochondria (36) in rat liver, one can calculate that there should be about 3 x 10\(^8\) of each of these enzyme molecules/microorganism. If these enzymes do associate into an insoluble aggregate in vivo, as suggested by several experiments (3, 15), the size of the aggregates will be considerably smaller than 1 μm. Nevertheless, the properties important for substrate channeling, namely the local concentrations of both enzymes and their ratio to local oxalacetate concentrations, should be very similar for the in vitro and in vivo systems. Thus, it is reasonable to assume that substrate channeling of oxalacetate can occur in mitochondria.

Just how important or necessary substrate channeling is in the mitochondrial under normal or abnormal conditions is uncertain due, in large part, to experimental difficulties in estimating free substrate and hydrogen ion concentrations in this organelle. It is helpful, however, to consider some implications and possible advantages of substrate channeling. First, we should emphasize that substrate channeling does not preclude physiologically significant net fluxes of oxalacetate between local and bulk phase. Therefore, we might define the efficiency of oxalacetate channeling from the malate dehydrogenase, at any instant, as the fraction of the total flux producing citrate that is derived from oxalacetate produced locally by the dehydrogenase reaction. This efficiency can be changed by anything that reduces the dehydrogenase reaction rate below that of citrate production, e.g. an increased NADH/NAD redox ratio, changes in anaplerotic rates of oxalacetate production or utilization, or changes in the composition of the dehydrogenase-synthase complex, e.g. the replacement of the synthase by aspartate aminotransferase (8). Thus, substrate channeling efficiency can be a dynamically changing response to conditions. New mechanisms of metabolic regulation are also provided, by which oxalacetate flux can be directed to and from other reactions.

If the mitochondrial matrix and enzymes were a homogeneous phase, free oxalacetate concentrations would have to be very low because of the unfavorable equilibrium of the malate dehydrogenase reaction and its unfavorable rate from near-equilibrium conditions.\(^2\) As Srere has discussed (37), such a low value of free oxalacetate would predict citrate synthase reaction rates below those measured (as oxygen consumption) for the Krebs cycle. More recent data might suggest higher free oxalacetate concentrations, and there are experimental uncertainties in these estimates. However, our evaluation of current data still suggests that there are metabolic conditions (e.g. high NADH/NAD ratios) and tissues in which a homogeneous distribution of oxalacetate would be unable to maintain the observed Krebs cycle rate. Substrate channeling can, in principle, circumvent some of these constraints of homogeneous catalysis. For mechanisms in which metabolites are directly transferred from one enzyme to the next, the local or bulk concentrations of the unbound intermediates are irrelevant to the reaction rates. If oxalacetate must dissociate from the dehydrogenase before reacting with the synthase, local high oxalacetate concentrations could maintain high rates for the synthase reaction. The unfavorable effects of locally high oxalacetate concentrations on the dehydrogenase reaction could be reduced by maintaining the local NADH/NAD ratio low; the latter might be achieved through the association of malate dehydrogenase with complex I (9).

In summary, we see that molecular compartmentation achieved by substrate channeling can remove constraints from competing reaction equilibria and rates and provide new ways of metabolic regulation. Transitions from one steady state to another could also be accomplished faster with substrate channeling, since a larger quantity of metabolite is required to cause the same change in a bulk phase concentration as in a smaller local environment. However, the demonstration of substrate channeling in vitro and recognition of its possible advantages, although suggestive, is not conclusive evidence of its importance in vivo.

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