Regulation of Aminotransferase-Glutamate Dehydrogenase Interactions by Carbamyl Phosphate Synthase-I, Mg\(^{2+}\) plus Leucine Versus Citrate and Malate*

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Leonard A. Fahien§, Edward H. Kmietok‡, Gebretaweos Woldegorgis†, Merle Evenson¶, Earl Shrago¶, and Margaret Marshall†

From the Departments of ‡Pharmacology, ¶Nutritional Sciences, and ¶¶Medicine, University of Wisconsin, Madison, Wisconsin 53706

Citrate, malate, and high levels of ATP dissociate the mitochondrial aspartate aminotransferase-glutamate dehydrogenase complex and have an inhibitory effect on the latter enzyme. These effects are opposed by Mg\(^{2+}\), leucine, Mg\(^{2+}\) plus ATP, and carbamyl phosphate synthase-I. In addition, Mg\(^{2+}\) directly facilitates formation of a complex between glutamate dehydrogenase and the aminotransferase and displaces the aminotransferase from the inner mitochondrial membrane which could enable it to interact with glutamate dehydrogenase in the matrix.

Zn\(^{2+}\) also favors an aminotransferase-glutamate dehydrogenase complex. It, however, is a potent inhibitor of and has a high affinity for glutamate dehydrogenase. Leucine, however, enhances binding of Mg\(^{2+}\) and decreases binding of and the effect of Zn\(^{2+}\) on the enzyme. Thus, since both metal ions enhance enzyme-enzyme interaction and Zn\(^{2+}\) is more potent inhibitor, the addition of leucine in the presence of both metal ions results in activation of glutamate dehydrogenase without disruption of the enzyme-enzyme complex. Furthermore, the combination of leucine plus Mg\(^{2+}\) produces slightly more activation than leucine alone.

These results indicate that leucine, carbamyl phosphate synthase-I, and its substrate and cofactor, ATP and Mg\(^{2+}\), operate synergistically to facilitate glutamate dehydrogenase activity and interaction between this enzyme and the aminotransferase. Alternatively, Krebs cycle intermediates, such as citrate and malate, have opposing effects.

According to recent work, there is integration and interaction between mitochondrial aspartate aminotransferase (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1) and enzymes of the Krebs cycle. When glutamate is presented to or generated from glutamine in liver or tumor mitochondria, the first step in its oxidation is transamination with oxalacetate, provided by the malate dehydrogenase (L-malate:NAD\(^+\) oxidoreductase, EC 1.1.1.37) reaction, followed by metabolism of the generated \(\alpha\)-ketoglutarate (by the Krebs cycle) to restore the level of oxalacetate (1, 2).

The aminotransferase, citrate synthase (citrate oxalacetate lyase, EC 4.1.3.7), and malate dehydrogenase all have a high affinity for the inner surface of the inner mitochondrial membrane (3). Furthermore, there is evidence that complexes can be formed between these three enzymes (4–6). Thus, it seems possible that, as proposed, these enzymes react in a sequence as a multi-enzyme cluster (2–6).

Mitochondrial aspartate aminotransferase can also form complexes with glutamate dehydrogenase (L-glutamate: NAD(P)+ oxidoreductase (deaminating), EC 1.4.1.3) and carbamyl phosphate synthase-I (EC 6.3.4.16) (7–13). Furthermore, carbamyl phosphate synthase-I does not displace but can enhance aminotransferase-glutamate dehydrogenase interactions, even in the presence of substrates of these enzymes (12, 13). This, plus the fact that, in the liver mitochondrial matrix, glutamate dehydrogenase can be cross-linked to carbamyl phosphate synthase-I (14), suggests that a complex can also be formed among these three enzymes. These interactions are specific and could be physiologically significant because they take place at concentrations less than those present in vivo. Indeed, carbamyl phosphate synthase-I is essentially present only in liver mitochondria (15), where it is the most abundant mitochondrial protein, and its level has been estimated to be as high as 0.4–1.0 mM (16–18). Consequently, this enzyme could play a structural role by regulating interaction between glutamate dehydrogenase and the aminotransferase when the latter enzyme is involved in NH\(_2\)-aspartate metabolism. Since generation of carbamyl phosphate is a unique function of liver mitochondria, this regulatory or structural role could be the reason why carbamyl phosphate synthase-I is present in extremely high levels.

While the aminotransferase-glutamate dehydrogenase complex is stable in the presence of substrates (13) of these enzymes, two Krebs cycle intermediates, citrate and malate, as well as high levels of ATP dissociate this complex (12, 13). This is of interest in view of the fact that malate and citrate play key roles in determining whether oxalacetate is converted into citrate, malate, or aspartate. These and other results suggest structural organization of the aminotransferase in opposing heteroenzyme clusters and regulation of these complexes by substrates and allosteric modifiers of the constituent enzymes. Indeed, this might be the case in tumor mitochondria where glutamate oxidation leads to the production of a high level of citrate (2, 19).

The effects of metal ions on these interactions have not been studied. This could be of interest because Mg\(^{2+}\) is a cofactor of the carbamyl phosphate synthase-I reaction, Zn\(^{2+}\) is a potent inhibitor of both carbamyl phosphate synthase-I (20) and glutamate dehydrogenase (21, 22), and it would be expected that metal ions would modify the effects of citrate on enzyme-enzyme interactions. Also, as mentioned above,
the aminotransferase has a high affinity for the inner mitochondrial membrane (3, 23). This might restrict the amount of this enzyme available to interact with glutamate dehydrogenase and carbamyl phosphate synthase-I in the liver mitochondrial matrix. Therefore, in this paper, we have studied in more detail the effects of citrate, malate, and metal ions on enzyme-enzyme interactions and binding of the aminotransferase to the inner mitochondrial membrane.

In some experiments, enzyme-enzyme interaction was determined by measuring coprecipitation of the enzymes in polyethylene glycol. These conditions might mimic the mitochondrial matrix with polyethylene glycol substituting for the noninteracting proteins (24). Polyethylene glycol does not primarily promote complex formation but mainly precipitates complexes which are found in its absence (25). Consequently, we have found that results obtained in polyethylene glycol are consistent with those obtained by other techniques including: enzyme immobilized on Sepharose (10), divalent cross-linkers (9), and gel equilibrium (7). However, because of conceivable limitations of performing experiments in polyethylene glycol, results are also presented which were obtained in its absence.

**MATERIALS AND METHODS**

**Enzymes and Reagents**—Bovine and rat liver mitochondrial aspartate aminotransferase and glutamate dehydrogenase were prepared with previously described methods (26–29). Rat liver carbamyl phosphate synthase-I was prepared with some modifications of a previously described method (30). Pig heart mitochondrial malate dehydrogenase and citrate synthase were obtained from Boehringer Mannheim and Sigma, respectively. The latter was also the source of bovine serum albumin. All of the enzymes were over 99% pure as estimated from electrophoresis on Sepharose (10), divergent cross-linkers (9), and gel equilibrium (7). The latter, however, because of conceivable limitations of performing experiments in polyethylene glycol, results are also presented which were obtained in its absence.

**Concentration of Enzymes**—Protein concentrations were determined by measuring the absorbance at 280 nm as described previously (13). The concentration or amount of enzyme was converted from milligrams/milliliter or micrograms to milliunits or millicons by the total amount of enzyme units incubated.

**Coprecipitation of Enzymes**—The results obtained were essentially as described previously (4, 18). Solutions of enzymes plus ligands were incubated in 4% (w/v) solution of 0.2 M potassium phosphate, 0.1 mM EDTA, pH 7.0. The solutions were then removed, and the precipitated enzyme was then determined by measuring coprecipitation of the enzymes in the supernatant or the precipitate by the total amount of enzyme units incubated.

**Preparation and Experiments with Inverted Inner Mitochondrial Membranes**—Rat liver inverted inner mitochondrial membranes were prepared essentially as described previously (3, 38). The amounts of membranes or organelles are given in terms of micrograms or milligrams of proteins. The inner membranes were washed extensively with 2 mM HEPES buffer, pH 7.0. For the binding studies, enzyme samples and membranes (1.7 mg/ml) were incubated for 15 min at 0 °C in (1 ml) 2 mM HEPES buffer, 0.5 mM dithiothreitol, pH 6.9. The membranes were then sedimented and washed with the same buffer. The final pellet was then resuspended in 1 ml of the above buffer, as described previously (3), and enzyme activities in the different fractions were measured. Over 90% recovery of added enzyme activity was observed. Thus, the amount of bound protein could be obtained from multiplying the fraction of enzyme units in either the supernatant or the precipitate by the total amount of enzyme units incubated.

**Treatment of Enzymes with Chelex 100**—Carbamyl phosphate synthase-I and glutamate dehydrogenase were dialyzed versus 0.2 M potassium phosphate, 0.1 mM EDTA, pH 7.0, and then versus this buffer (minus EDTA) which had been chromatographed on Chelex. Polyethylene glycol (6000) was obtained from Fisher and prepared as a 40% (w/v) solution in 0.02 M potassium phosphate, pH 7.0. Other substrates, enzymes, coenzymes, and reagents were obtained from Sigma. Stock solutions of all reagents used in assays were adjusted to the pH of the assays and prepared as sodium salts. Solutions of coenzyme were prepared fresh daily.

**Results and Discussion**

Effect of Mg²⁺ on Aminotransferase-Glutamate Dehydrogenase Interaction—As shown in Fig. 1 (curves A and B), Mg²⁺ increases the amount of glutamate dehydrogenase associated with the aminotransferase. This is actually an increase in enzyme-enzyme interaction because Mg²⁺ does not markedly enhance precipitation of either enzyme alone (Fig. 1, curves E and F). Furthermore, this effect is not due to increasing ionic strength, which in general tends to decrease interaction with ATP (5 mM), NH₄CO₃ neutralized with CO₂ (50 mM), N-acetylglutamate (5 mM), magnesium acetate (10 mM), ornithine (2 mM), and an excess of ornithine trimethylamine as described previously (37). The assays were performed in 50 mM triethanolamine, pH 7.0, at 30 °C.

**Turbitude Experiments**—These experiments were performed in an Amino-Bowman spectrofluorometer equipped with an E polarizer. The instrument was adjusted prior to each experiment so that a 0.5-mg/ml solution of blue dextran would read 195.

**Turbidity Experiments**—These experiments were performed in an Amino-Bowman spectrofluorometer equipped with an E polarizer. The instrument was adjusted prior to each experiment so that a 0.5-mg/ml solution of blue dextran would read 195.

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Mg^{2+} increases the turbidity of glutamate dehydrogenase alone (Table I) and inhibits this enzyme (not shown) over the same concentration range required for enhancement of enzyme-enzyme interaction. Mg^{2+} has no effect on the turbidity (Table I) or the activity of the aminotransferase (not shown). Thus, Mg^{2+} is bound to glutamate dehydrogenase, and this apparently increases enzyme-enzyme interaction.

**Interactions with Malate Dehydrogenase**—In both the presence (not shown) and absence of polyethylene glycol (Table I), Mg^{2+} had considerably less effect on the complex between malate dehydrogenase and glutamate dehydrogenase. Therefore, Mg^{2+} is another factor (5, 18) which favors interaction between glutamate dehydrogenase and the aminotransferase over interactions between glutamate dehydrogenase and malate dehydrogenase.

**Specificity of Metal Ions**—The effects of Mg^{2+} on aminotransferase-glutamate dehydrogenase interactions and glutamate dehydrogenase activity are not specific for this divalent cation. Mn^{2+} (not shown) and Ca^{2+} have effects comparable to Mg^{2+}. Zn^{2+} is effective at quite low levels where neither Mg^{2+} nor Ca^{2+} have an effect (Tables I and II and Fig. 2), and it increases the amount of both enzymes in the complex, while Mg^{2+} and Ca^{2+} mainly enhance the amount of glutamate dehydrogenase associated with aminotransferase (Table II). Zn^{2+}, as the other metal ions, did not precipitate either enzyme alone (Table II) and did not alter aminotransferease activity (not shown).

The effects of metal ions on enzyme-enzyme interaction and glutamate dehydrogenase activity (Tables I and II and Fig. 2) were observed in the absence of EDTA, in experiments performed with enzymes, and other constituents of the assays which were treated with Chelex. Thus, the effect of metal ions does not result from displacing EDTA from the enzyme. In addition, treating both enzymes with Chelex (not shown) and adding EDTA to Chelex-treated enzymes do not markedly alter enzyme-enzyme interaction (Table II) or glutamate dehydrogenase activity (not shown). The slight observed effect of EDTA could result from binding of this chelator to glutamate dehydrogenase (21, 22).

The glutamate dehydrogenase used in these experiments was crystallized four times in 0.1 mM EDTA, dialyzed versus and, as shown in Fig. 1 (curves C and D), NaCl has no significant effect.

This effect of Mg^{2+} on enzyme-enzyme interaction can also be seen in the absence of polyethylene glycol. Thus, as shown in Table I (lines 1-3), in the absence of polyethylene glycol, the turbidity of the mixture of the two enzymes is considerably greater than the sum of the turbidity of either enzyme alone, and 1 mM Mg^{2+} increases this difference. That is, even though Mg^{2+} slightly increases the turbidity of glutamate dehydrogenase alone (but not the aminotransferase alone), this influence is considerably less than that produced by Mg^{2+} in the presence of both enzymes.
Enzyme-Enzyme Interaction

**Fig. 2.** Plot of specific activity of glutamate dehydrogenase versus concentration of metal ions. These experiments were performed in the presence of MgCl₂ (curve A), CaCl₂ (curve B), or ZnCl₂ (curve C) in 1.0 mM TPN, 5.0 mM glutamate plus 0.02 mM potassium phosphate, pH 7.0. The concentration of enzyme in most experiments was 10 μg/ml. Specific activity is expressed in units of change in optical density at 340 nm/min/mg of enzyme. In these experiments, enzyme, buffer, TPN, and glutamate were all chromatographed on Chelex prior to use.

![Graph](image-url)

**Fig. 3.** Plot of velocity (change in optical density at 340 nm/min) versus concentration of leucine. These experiments were performed in 1.0 mM TPN, 5.0 mM glutamate in 0.02 mM potassium phosphate, 10 μM EDTA, pH 7.0. The concentration of glutamate dehydrogenase was 8 μg/ml. Other additions were 1 mM MgCl₂ (curve A), 1 mM MgCl₂ plus 5 μM ZnCl₂ (curve B), no additions (curve C), and 8 μM ZnCl₂ (curve D). Buffer, enzyme, and substrates were treated with Chelex prior to use in these experiments.

![Graph](image-url)

**Table III**

| Enzyme(s) | Additions | Enzyme precipitated |
|-----------|-----------|---------------------|
| Both      | None      | 0.49 0.35           |
| Both      | Leucine (2 mM) | 0.35 0.35         |
| Both      | ZnCl₂ (20 μM) | 0.52 0.51          |
| Both      | ZnCl₂ (20 μM), leucine (2 mM) | 0.70 0.45 |
| Both      | MgCl₂ (1 mM) | 0.76 0.42          |
| Both      | MgCl₂ (1 mM), leucine (2 mM) | 0.76 0.42         |
| Both      | MgCl₂ (1 mM), ZnCl₂ (20 μM) | 0.73 0.68         |
| Both      | Lecine (2 mM), MgCl₂ (1 mM), ZnCl₂ (20 μM) | 0.97 0.72 |
| GDH       | Leucine (2 mM), MgCl₂ (1 mM), ZnCl₂ (20 μM) | 0.10             |
| Asp-AT    | Leucine (2 mM), MgCl₂ (1 mM), ZnCl₂ (20 μM) | 0.03             |

**Effect of leucine and metal ions on enzyme-enzyme interaction**

In these experiments, 1.8 nmol of glutamate dehydrogenase (GDH) and 1.1 nmol of mitochondrial aspartate aminotransferase (Asp-AT) were incubated either alone or together with 1.0 mM TPN plus the indicated additions in 1.0 ml of polyethylene glycol (14%, w/v), 14 mM potassium phosphate, 10 μM EDTA, pH 7.0, at 25°C. After 20 min, the solutions were centrifuged and assayed for enzyme activity as described under "Materials and Methods." Buffer, TPN, and enzymes were treated with Chelex prior to use in the experiments.

0.1 mM EDTA, and then dialyzed extensively versus Chelex-treated phosphate buffer (see "Materials and Methods"). When glutamate dehydrogenase, prepared by this method, was assayed with neutron activation (both short- and long-term irradiation schedules) and atomic absorption spectroscopy, only 0.3 eq of Ca²⁺/enzyme polypeptide chain was found. The former method screens for 50–60 metals including Zn²⁺. Thus, it can be concluded that only a small amount of Ca²⁺ was associated with the glutamate dehydrogenase used in these experiments.

**Effect of Leucine**—While metal ions enhance enzyme-enzyme interaction, they also reversibly inhibit glutamate dehydrogenase (Fig. 2). However, leucine can reverse this inhibition (Fig. 3, curves A and D). Also, Mg²⁺ slightly enhances activation of this enzyme by leucine in either the presence or absence of Zn²⁺ (Fig. 3, curves A and B versus C). That is, the rate in the presence of Mg²⁺ and leucine is actually higher than it is in the presence of leucine alone.

While leucine can reverse inhibition by and even activate glutamate dehydrogenase in the presence of metal ions, it does not decrease the ability of metal ions to enhance enzyme-enzyme interaction (Table III). Consequently, both glutamate dehydrogenase activity and enzyme-enzyme interaction are optimal in the presence of leucine and metal ions. This would not be the case if leucine were functioning as a chelator which removed the metal ion from glutamate dehydrogenase or if leucine competitively displaced the metal ion from this enzyme. Furthermore, ornithine and alanine, which have a low affinity for glutamate dehydrogenase, do not decrease inhibition by Zn²⁺ (Fig. 4, curves C and D), even though the latter is even a better chelator of Zn²⁺ than leucine (39). Alternatively, valine, which is a considerably poorer chelator of Zn²⁺ than alanine (39) but is bound to the same site on bovine glutamate dehydrogenase as leucine (40), decreases inhibition but has less of an effect than leucine (Fig. 4, curve B).

While the combination of leucine plus metal ions results in optimal enzyme-enzyme interaction, there is no precipitation of either of these enzymes alone under these conditions (Table III). In these experiments (Table III and Figs. 3 and 4), Ca²⁺ and Mg²⁺ had similar effects.

According to the above results, leucine is not competitive with metal ions for glutamate dehydrogenase. Consequently, Mg²⁺ facilitates activation of glutamate dehydrogenase by leucine. This synergy between leucine and Mg²⁺ is also shown in Fig. 5. It can be seen that both leucine and Mg²⁺ alone
probably competitive with Zn\(^{2+}\) because it, unlike leucine, does not prevent dissociation of glutamate dehydrogenase by saturating concentrations of Zn\(^{2+}\) (Fig. 5, right, curve A). However, Mg\(^{2+}\) is added in the presence of both metal ions, the effect of Zn\(^{2+}\) is decreased, and glutamate dehydrogenase activity and polymerization are restored to the level observed in the absence of Zn\(^{2+}\) (Figs. 3 and 5, curve A versus B). Similarly, when Zn\(^{2+}\) is added in the presence of leucine plus Mg\(^{2+}\), it produces little dissociation of the enzyme (Fig. 5, right, curve A). In these experiments (Fig. 5), the effects of Ca\(^{2+}\) and Mg\(^{2+}\) were again quite similar.

The concept that leucine decreases binding of Zn\(^{2+}\) is consistent with equilibrium dialysis experiments. The addition of 1.0 mM leucine to these experiments (see "Materials and Methods") decreased the amount of Zn\(^{2+}\) bound to glutamate dehydrogenase from 0.9 to 0.6 eq/enzyme polypeptide chain.

Effect of ATP, Malate, and Citrate—ATP and other purine nucleotides have no significant effect on aminotransferase activity (not shown). However, GTP and ADP are bound to nonidentical but overlapping sites on glutamate dehydrogenase in the vicinity of the leucine site. At these sites, ADP activates and GTP inhibits enzyme activity (40–42). GTP and higher levels of ADP are also bound to additional sites on this enzyme (41, 43). The effects of ATP, which is a substrate of carbamyl phosphate synthase-I, have not been studied in much detail. As shown in Fig. 6 (curve A) and Fig. 7 (curves C and F), ATP at low (100 \(\mu\)M) levels activates glutamate dehydrogenase but has little effect on enzyme-enzyme interaction. At higher levels, it produces less activa-

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The nonlinearity of double-reciprocal plots of velocity Furthermore, free Mg\(^{2+}\) and a high level of free ATP have ADP. TPN, which is not bound to the allosteric site ADP was competitive with DPN

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gives linear double-reciprocal plots, and we have found that both the active and ADP allosteric sites. This accounts for the nonlinearity of double-reciprocal plots of velocity versus DPN concentration and, possibly, the competition with ATP. TPN, which is not bound to the allosteric site (42), gives linear double-reciprocal plots, and we have found that at pH 7.0, TPN and high levels of ATP were not competitive (not shown). These results indicate that the second ADP site is not the active site. Furthermore, even a high (1 mM) level of DPNH (which is bound to both an active and a substrate inhibition site (41, 42)) does not itself markedly alter either enzyme-enzyme interaction or the effect of ATP on these interactions (Fig. 7, curves D and E).

The predominant form of ATP in vivo and the actual substrate of carbamyl phosphate synthase-I is Mg-ATP (44). As shown in Fig. 6 (curve B) and Fig. 7 (curves A and B), Mg\(^{2+}\) decreases the ability of ATP to dissociate the enzyme-enzyme complex without preventing ATP from activating glutamate dehydrogenase. This is apparently because a high level of free ATP is required for dissociation of the complex, while only a low level is required for enzyme activation. Furthermore, free Mg\(^{2+}\) and a high level of free ATP have opposing effects on the stability of the enzyme-enzyme complex, while neither (in the presence of 100 \(\mu\)M EDTA) has a marked effect on enzyme activity (Fig. 6). Mg-ATP is apparently not bound to glutamate dehydrogenase (45).

The effects of ATP on enzyme-enzyme interaction can also be seen in the absence of polyethylene glycol. ATP alone decreases the ratio of the turbidity of the combination of the two enzymes to the sum of the turbidity of each enzyme alone (Fig. 8, curve E). This is because ATP produces a greater decrease in the turbidity of aminotransferase-glutamate dehydrogenase (Fig. 8, curve C) than it does in the turbidity of glutamate dehydrogenase alone (Fig. 8, curve F). In the presence of Mg\(^{2+}\), ATP increases this ratio (Fig. 8, curve B) because it has considerably less of an effect on the turbidity of the mixture of the two enzymes (Fig. 8, curve A) than it does on the turbidity of Mg\(^{2+}\) plus glutamate dehydrogenase alone (Fig. 8, curve D). This is again consistent with ATP dissociating Mg\(^{2+}\) facilitating association of the enzyme-enzyme complex. Neither ATP nor Mg\(^{2+}\) alters the turbidity of the aminotransferase alone (Fig. 8, curve G).

Malate is bound to the active site of glutamate dehydrogenase and decreases interaction between this enzyme and the aminotransferase (46). Even though leucine or low levels of ATP or ADP are bound to allosteric sites, they antagonize the effects of malate on this enzyme and enzyme-enzyme interaction (46). Consequently, while low levels of ATP have no effects on enzyme-enzyme interaction in the absence of malate, in the presence of DPNH or TPNH plus malate, they enhance these interactions apparently by displacing malate from glutamate dehydrogenase (Table IV, lines 3 and 4). Malate has less effect in the presence of TPN (46) and, under

**Fig. 7.** Plot of enzyme precipitated versus concentration of ATP. In these experiments, 1.8 nmol of glutamate dehydrogenase and 2.2 nmol of mitochondrial aspartate aminotransferase were incubated for 20 min in 1.0 ml of polyethylene glycol (14%, w/v), 14 mM potassium phosphate, 0.1 mM EDTA, pH 7.0, at 25 °C. This time, the solutions were centrifuged for 10 min at 25 °C, and the precipitate was assayed for either glutamate dehydrogenase (curves A, C, and D) or aminotransferase (curves B, E, and F) as described under "Materials and Methods." Additions to the incubations were: 1.0 mM TPN plus 1 mM MgCl\(_2\) (curves A and B), TPN alone (curves C and F), and 1.0 mM DPNH (curves D and E).

**Fig. 8.** Plot of enzyme turbidity versus concentration of ATP. In these experiments, ATP was added after a 5-min incubation to glutamate dehydrogenase plus mitochondrial aspartate aminotransferase in either the presence (curve A) or absence (curve C) or 1 mM MgCl\(_2\) or glutamate dehydrogenase alone in either the presence (curve D) or absence (curve F) of 1 mM MgCl\(_2\). Curve G shows the results obtained when ATP is added to the aminotransferase in either the presence or absence of 1 mM MgCl\(_2\). Plots of the ratio of the turbidity of the mixture of the two enzymes to the sum of the turbidity of either enzyme alone in either the presence or absence of 1 mM MgCl\(_2\) are shown in Curves B and E, respectively. The concentration of glutamate dehydrogenase and aminotransferase was, respectively, 18 and 22 \(\mu\)M. These experiments were performed in 10 mM potassium phosphate, 0.1 mM EDTA, pH 7.0, at 25 °C.
polyethylene glycol (14%, w/v), 14 mM potassium phosphate, 0.1 mM transferase (Asp-AT) was incubated with 1.8 nmol of glutamate
are not decreased by α-ketoglutarate (Table IV, line 5 versus
less than 10% of the activity (not shown). Citrate, unlike malate, is probably not bound to the active site of glutamate dehydrogenase because it markedly decreases the activity of this enzyme in the presence of ATP or leucine (Fig. 11, left, curve A versus E, and right, curve B versus D). This apparently does not result from binding of citrate to the overlapping ATP-leucine site, because citrate does not completely eliminate the effect of these activators (Fig. 11) and, in the presence of DPNH, low levels of ATP do not decrease dissociation of the aminotransferase-glutamate dehydrogenase complex by citrate (Table IV, line 5 versus 6). Therefore, citrate is apparently bound to a site on glutamate dehydrogenase which is distal from both the active and ATP allosteric sites. Binding of citrate to this site has little effect on enzyme activity but decreases activation by ATP or leucine and decreases enzyme-enzyme interaction.

As shown in Fig. 10 (bottom), citrate decreases enzyme-enzyme interaction only when its concentration exceeds that of Mg++. Furthermore, Mg++ enhances glutamate dehydrogenase activity in the presence of citrate plus either ATP or leucine (Fig. 11, left, curve B versus E, and right, curve C versus D).

It is unlikely that the effects of citrate, malate, or high levels of ATP in polyethylene glycol result from these ligands increasing the solubility of either enzyme alone rather than dissociating the enzyme-enzyme complex. Under these experimental conditions and even when the level of enzyme is 5-fold higher, both glutamate dehydrogenase alone and the aminotransferase alone are essentially completely soluble in polyethylene glycol (less than 5% precipitated; Refs. 5, 13,
and 18), and these ligands did not increase the solubility of either enzyme alone (not shown). In addition, it has been shown that, in the absence of polyethylene glycol, malate decreases cross-linking between these two enzymes (46), and ATP decreases the turbidity of the enzyme-enzyme complex (Fig. 8).

Citrate and high levels of ATP are similar in that both decrease enzyme-enzyme interaction, and this is reversed by Mg$^{2+}$. Also, both decrease activation of glutamate dehydrogenase by low levels of ATP, both are chelators, and both markedly increase ionic strength. However, it is unlikely that chelation plays a major role in the effect of citrate and ATP on either glutamate dehydrogenase activity or enzyme-enzyme interaction in the absence of added metal ions. This is because, as mentioned above, only a small amount of Ca$^{2+}$ is associated with glutamate dehydrogenase. Furthermore, both citrate and high levels of ATP slightly decrease glutamate dehydrogenase activity, while metal ions inhibit this enzyme.

Thus, if citrate and high levels of ATP functioned as chelators, they would be expected to be activators. In addition, treating both enzymes and buffer with Chelex did not markedly decrease enzyme-enzyme interaction (not shown), and added EDTA had little effect on Chelex-treated enzymes (Table II). Furthermore, isocitrate, which is a 10-fold poorer chelator of Mg$^{2+}$ than citrate (48), has an effect almost equal to that of citrate (Table IV). Finally, dialyzing both enzymes versus 4 mM citrate had less of an effect on enzyme-enzyme interaction than adding citrate (Table V).

Citrate and high levels of ATP also do not decrease enzyme-enzyme interaction and inhibit glutamate dehydrogenase as a result of increasing ionic strength. NaCl, in comparable ionic strengths, has considerably less of an effect than citrate on either glutamate dehydrogenase activity in the presence of ATP or leucine or enzyme-enzyme interaction (Figs. 11 and 12). Furthermore, in the absence of coenzyme, 1 mM citrate (ionic strength, $5 \times 10^{-3}$) produces a greater decrease in enzyme-enzyme interaction than 4 mM malate (ionic strength, $12 \times 10^{-3}$) (Table IV). Also, citrate itself does not markedly inhibit glutamate dehydrogenase in the absence of leucine or ATP (Figs. 11 and 12), and 1 mM levels of DPNH, TPNH, TPN, and DPN have considerably less effect than ATP on enzyme-enzyme interaction (Table IV).

Effect of Carbamyl Phosphate Synthase-I—According to these and previous results, both Mg$^{2+}$ and carbamyl phosphate synthase-I retard the decrease in aminotransferase-glutamate dehydrogenase interaction caused by citrate or high levels of ATP but not that caused by malate (12, 13). This suggests...
enzy~m~e-enzyme interaction. The buffer was chromatographed on Chelex, and all li-
gands were dissolved in the Chelex-treated buffer. Where indicated, enzyme was dialyzed twice versus 500 ml of 4 mM citrate in the above phosphate buffer.

| Additions or other conditions | Enzyme precipitated | mmol |
|-----------------------------|---------------------|------|
| None                        | GDH                 | 0.78 |
| Asp-AT dialyzed versus 4 mm citrate | Asp-AT     | 0.78 |
| GDH dialyzed versus 4 mm citrate | CPS       | 0.60 |
| 4 mm citrate                | CS                  | 0.50 |
| CPS, 4 mm citrate           | A                   | 0.39 |
| Chelex, 4 mm citrate        | B                   | 0.55 |
| CS, 4 mm citrate            | C                   | 0.18 |
| 4 mm Citrate, 0.5 mm MgCl₂  | D                   | 0.07 |
| Chelex, CPS                 |                     | 1.1  |

FIG. 12. Plot of enzyme precipitated versus increase in ionic strength. In these experiments, 1.8 nmol of glutamate dehydrogenase and 2.2 nmol of mitochondrial aspartate aminotransf~erase (Asp-AT) were incubated in 1 ml of 14% (w/v) polyethylene glycol, 1 mM TPN, 14 mM potassium phosphate, 0.1 mM EDTA, pH 7.0, at 25 °C. After 20 min, the solutions were centrifuged and assayed as described under “Materials and Methods.” Chelex-treated enzymes refers to enzymes which had been either chromatographed on a column of Chelex or dialyzed versus Chelex as described under “Materials and Methods.” The buffer was chromatographed on Chelex, and all ligands were dissolved in the Chelex-treated buffer. Where indicated, enzyme was dialyzed twice versus 500 ml of 4 mM citrate in the above phosphate buffer.

that this effect of carbamyl phosphate synthase-I results from release of a metal ion associated with this enzyme. However, 2 μM of this enzyme enhance aminotransferase-glutamate dehydrogenase interaction even in the presence of cheletors such as 4 mM citrate and 0.1 mM EDTA (Table V, line 4 versus 5). Under these conditions, even 0.5 mM Mg²⁺ has no effect on aminotransferase-glutamate dehydrogenase interaction (Table V, line 4 versus 8). Furthermore, treating carbamyl phosphate synthase-I with Chelex does not decrease but slightly enhances its effect (Table V, line 5 versus 6). Therefore, a metal ion associated with carbamyl phosphate synthase-I is unlikely to be responsible for its ability to enhance aminotransferase-glutamate dehydrogenase interactions.

The aminotransferase can also form a complex with carbamyl phosphate synthase-I (13). However, enhancement of aminotransferase-glutamate dehydrogenase interaction by carbamyl phosphate synthase-I is apparently not the result of nonspecific absorption of glutamate dehydrogenase to the aminotransferase-carbamyl phosphate synthase-I complex (13). If this were the case, then it would be more likely to take place in the presence of malate than citrate, because only the latter markedly decreases interaction between carbamyl phosphate synthase-I and the aminotransferase (12, 13). Another reason for believing that there is interaction among all three enzymes is that considerably less amounts of anionic proteins, other than glutamate dehydrogenase, associate with these two enzymes (13), and high levels of citrate synthase have considerably less of an effect than carbamyl phosphate synthase-I in enhancing aminotransferase-glutamate dehydrogenase interactions (Table V, line 5 versus 7). Thus, these plus results, which demonstrate cross-linking between glutamate dehydrogenase and carbamyl phosphate synthase-I in the mitochondrial matrix (14), suggest that a complex is formed between all three enzymes.

Binding to Inner Mitochondrial Membrane—It has previously been shown that mitochondrial malate dehydrogenase and aspartate aminotransferase have a high affinity for the inner surface of the inner mitochondrial membrane (3, 23). The binding process is saturable and specific in that a series of nonmatrix proteins, including cytosolic malate dehydrogenase and aspartate aminotransferase, have a low affinity for these vesicles (3). Citrate synthase was bound more poorly than the other two mitochondrial enzymes, and binding of this enzyme was decreased by malate dehydrogenase (3). In addition, as shown in Table VI (lines 1–4), the aminotransferase can displace malate dehydrogenase. These and previous results (3) indicate that there is a specific site on the membrane for these enzymes and the order of specificity is: aminotransferase, malate dehydrogenase, and citrate synthase. Glutamate dehydrogenase has a low affinity for the membrane, and its binding is also slightly decreased further by the aminotransferase (Table VI, lines 5–6). Carbamyl phosphate synthase-I is essentially not bound (not shown). Only a trace (less than 1%) amount of these enzymes was associated with the membrane in the absence of added enzymes.

As shown in Table VI (lines 2 and 7), 2 mM NaCl has little effect on binding of the aminotransferase to the membrane. This is similar to previous results (3) which indicated that 5–10 mM KCl or NaCl was required to produce a 2-fold decrease in binding of malate dehydrogenase. However, 1 mM MgCl₂ produces a marked decrease in binding of the aminotransferase (Table VI, line 9) and, in the presence of MgCl₂ and both aminotransferase and malate dehydrogenase, very little of either enzyme is bound (Table VI, line 9). Also, in the presence of MgCl₂, glutamate dehydrogenase further decreases binding of the aminotransferase, and this enzyme has a similar effect on binding of glutamate dehydrogenase (Table VI, line 10).
In these experiments, the effects of Ca\(^{2+}\) and Mg\(^{2+}\) were again similar.

**Physiological Significance**—In liver mitochondria, aspartate aminotransferase with glutamate dehydrogenase and carbamyl phosphate synthase-I regulate the synthesis of carbamyl phosphate and aspartate for urea. These reactions take place in a coordinated, integrated manner. The former enzyme. A high level of Mg\(^{2+}\) and carbamyl phosphate dehydrogenase activity. Thus, in the presence of leucine, the level of Mg\(^{2+}\) and carbamyl phosphate synthase-I are enhanced, and the opposing effects of citrate and malate mentioned above on the aminotransferase-glutamate dehydrogenase complex and on glutamate dehydrogenase activity can be associated with a higher affinity of this enzyme for the aminotransferase. Leucine enables metal ions to enhance aminotransferase-glutamate dehydrogenase interaction without inhibiting the latter enzyme. This gives leucine advantages over chelators which could also decrease inhibition by metal ions but, in the process, would also prevent the metal ion from enhancing enzyme-enzyme interaction. Thus, ATP could chelate, but this would prevent metal ions from enhancing enzyme-enzyme interaction. Furthermore, Mg\(^{2+}\) could competitively displace Zn\(^{2+}\) from ATP, which would leave Zn\(^{2+}\) available to inhibit glutamate dehydrogenase. Alternatively, leucine plus Mg\(^{2+}\) decreases the inhibitory effect of Zn\(^{2+}\).

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**Enzyme-Enzyme Interaction**

**Table VI**

*Binding of mitochondrial enzymes to inner mitochondrial membrane*

| Enzyme(s) added | Additions | Asp-AT nmol | MDH nmol | GDH nmol |
|-----------------|-----------|-------------|----------|----------|
| Asp-AT (11 nmol) | None      | 8.4 76      |          |          |
| Asp-AT (5.5 nmol) | None      | 5.1 93      |          |          |
| MDH (7 nmol)    | None      | 4.8 87      | 5.2 74   |          |
| MDH (7 nmol), Asp-AT (5 nmol) | None | 2.2 31      |          |          |
| GDH (18 nmol)   | None      | 4.0 72      | 4.3 24   |          |
| GDH (18 nmol), Asp-AT (5.5 nmol) | NaCl (2 mM) | 1.8 10      |          |          |
| Asp-AT (5.5 nmol) | MgCl\(_2\) (1 mM) | 4.7 85      |          |          |
| Asp-AT (5.5 nmol), MDH (7 nmol) | MgCl\(_2\) (1 mM) | 2.2 40      |          |          |
| GDH (18 nmol), Asp-AT (5.5 nmol) | MgCl\(_2\) (1 mM) | 1.5 27      | 5.0 28   |          |
| GDH (18 nmol)   | MgCl\(_2\) (1 mM) | 5.9 33      |          |          |

In these experiments, enzymes and inverted inner mitochondrial membranes were incubated for 15 min at 0 °C, sedimented, and washed, and the pellets and supernatant fractions were assayed for enzyme activity as described under "Materials and Methods." The abbreviations used are the same as given in the legend to Table I.
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