Communication

Binding of Citrate Synthase to Mitochondrial Inner Membranes*

(Received for publication, November 22, 1982)

Stanislaus F. D'Souza† and Paul A. Srere

From the Pre-Clinical Science Unit, Veterans Administration Medical Center, Dallas, Texas 75216 and Department of Biochemistry, University of Texas Health Science Center, Dallas, Texas 75235

Citrate synthase and other mitochondrial matrix proteins bind to the inner surface of the mitochondrial inner membrane. No binding was observed to the outer membrane or to the outer surface of the inner membrane.

When mitochondria are disrupted, and the membranes are removed by centrifugation, it is usually observed that all of the citrate synthase, aconitase, isocitrate dehydrogenase, fumarase, and malate dehydrogenase remain in the supernatant fraction (matrix) as soluble enzymes. Pyruvate dehydrogenase and α-ketoglutarate dehydrogenase complexes remain partially bound (1, 2) to the inner membrane, but can be removed easily (1, 2). Succinate dehydrogenase remains firmly bound to the inner membrane (3) and is sometimes considered an intrinsic membrane component, although some workers have shown its removal from the membrane by more drastic (but nondetergent) treatment (4).

There have been a number of conflicting reports concerning the association of matrix Krebs cycle enzymes with the mitochondrial inner membranes. Several groups have studied this problem using either differential digitonin extraction of mitochondria, or measuring the enzyme activity remaining with the isolated inner membrane fraction. Some malate dehydrogenase of pig heart and of chicken liver (5) has been reported to be fully bound to guinea pig heart inner membranes (7-9). By measuring the loss of latency of rat liver mitochondrial enzyme activities at different digitonin concentrations, Matlib and O'Brien (2) concluded that fumarase was located close to the inner membrane and malate dehydrogenase was located away from the inner membrane. Among the mitochondrial enzymes similar to fumarase were NAD-isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, and pyruvate dehydrogenase. Citrate synthase, NADP-isocitrate dehydrogenase, and aspartate aminotransferase behaved like malate dehydrogenase.

Wit-Peeters et al. (7) concluded that for a number of matrix enzymes, "no clear distinction can be made between membrane-bound and soluble matrix enzymes" but the enzymes only differed in the tightness of their binding to the inner membrane. On the other hand, Landriscina et al. (10) have reported that NADP-isocitrate dehydrogenase, aspartate aminotransferase, glutamate dehydrogenase, and NAD-isocitrate dehydrogenase are isolated in a membrane-free fraction. Thus, studies reported so far have not provided unequivocal evidence for or against specific interactions of these enzymes with the mitochondrial inner membrane.

Our studies of the inner membrane-matrix compartment of mitochondria have led us to postulate that an organization of Krebs cycle enzymes exists (11, 12). Histochemical studies (13) and cross-linking experiments (14) indicated that citrate synthase was located near the inner surface of the inner membrane. These results have prompted us to look for specific interactions between some of the so-called "soluble" Krebs cycle enzymes and the mitochondrial inner membrane. This communication provides evidence for a specific interaction between citrate synthase and two other Krebs cycle enzymes and the matrix surface of the inner membrane of rat liver mitochondria.

EXPERIMENTAL PROCEDURES

Materials—The following chemicals were obtained from the indicated sources: NADH, NADP, L-malic acid, DTNB, bovine serum albumin, yeast glucose-6-phosphate dehydrogenase, and yeast alcohol dehydrogenase from Sigma; oxalacetic acid from Calbiochem; CoA from P-L Biochemicals; pig heart mitochondrial malate dehydrogenase, pig heart citrate synthase from Boehringer-Mannheim; pig heart cytosolic malate dehydrogenase from Miles Laboratories; and trypsin from Worthington. Yeast citrate synthase was purified in this laboratory (15). CosAc was made from CoA and acetic anhydride (16). The enzymes, usually obtained as ammonium sulfate suspension were dialyzed against 2 mM Hepes buffer, pH 7.0.

Methods—Citrate (αι) synthase (EC 4.1.3.7) (17), malate dehydrogenase (EC 1.1.1.37) (18), fumarase (EC 4.2.1.2) (19), aspartate aminotransferase (EC 2.6.1.1) (20), glucose-6-phosphate dehydrogenase (EC 1.1.1.49) (21), and alcohol dehydrogenase (EC 1.1.1.1) (22) were assayed using the methods described elsewhere. Protein was measured by the method of Lowry et al. (23) using bovine serum albumin as the standard.

Mitochondria were isolated from livers of fasted (24 h) male Sprague Dawley rats (200-250 g, body weight) by the method described earlier (24, 25). Mitoplasts were prepared using digitonin as described by Greenawalt (26). Cytosolic protein was obtained by centrifugation of the liver homogenate at 144,000 × g for 60 min. The inverted inner membrane vesicles were obtained by sonication as described by Hackenbrock and Miller-Hammon (27). The amounts of membranes or organelles are given in terms of μg or mg of proteins. Matrix proteins were obtained by lysis of mitoplasts by freeze-thawing in an acetone-solid CO₂ mixture. Lysate was centrifuged at 144,000 × g for 60 min and the supernatant solution obtained was used as the matrix fraction. The inner membranes were washed extensively and cytosol and matrix fractions were dialyzed against 2 mM Hepes buffer, pH 7.0. For the binding studies, enzyme samples and membranes (700 μg) were incubated for 15 min at 0 °C in (400 μl) 2 mM Hepes buffer, 0.5 mM dithiothreitol, pH 7.0. The membranes were sedimented by centrifuging for 30 min in an Airfuge centrifuge at 30 psi and were washed with the same buffer. The final pellet was then resuspended

* This work was supported by Grant PCM 7904007 from the National Science Foundation and funding by the Veterans Administration Research Service. 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.

† Present address, Biochemistry and Food Technology Division, Bhabha Atomic Research Centre, Bombay 400 085, India.

T. C. Linn, personal communication.

---

The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid).
**RESULTS AND DISCUSSION**

When inner membrane vesicles are isolated by the sonication procedure used here (27), one obtains inside out vesicles whose sidedness can be estimated by measuring the activities of cytochrome c oxidase (EC 1.9.3.1) and cytochrome c reductase (EC 1.6.99.3) (28). Vesicles prepared in this laboratory were about 70% inside out. These vesicles contain no bound citrate synthase, malate dehydrogenase, fumarase, or aspartate amino transferase activity. When pure citrate synthase is added to this preparation, binding to these vesicles occurs and the process is saturable (Fig. 1). Binding of malate dehydrogenase occurs to a greater extent (Table I), and this process is also saturable (Fig. 1). In addition, it can be seen (Fig. 1) that malate dehydrogenase reduces the amount of citrate synthase that can be bound.

The binding of proteins to this preparation is specific in that a series of pure non-matrix proteins, including cytosolic malate dehydrogenase, does not bind to these vesicles (Table I). In addition, it has been noted that cytosolic rat liver proteins, as a group, bind less well than does matrix proteins (Table II). Our results also show that, whereas the isozymes of malate dehydrogenase, fumarase, and aspartate amino transferase present in the added matrix proteins bind quite well to the vesicles, the isozymes of these enzymes present in the cytosol fraction do not bind as well (Table II).

The binding is decreased by an increase in ionic strength (Fig. 2). In addition to KCl shown here, we have found that NaCl, potassium phosphate, Tris-acetate, and CaCl₂ also remove bound enzyme from the membranes with approximately the same efficiency. Binding is also decreased by an increase in the pH of the medium (Fig. 3). The same effect of pH change is observed when 2 mM Tris-HCl is used as a buffer in place of 2 mM Hepes. Since the ionic strength change with change of pH is opposite for these two buffers, then the effect is due to a change in pH and not due to change in ionic strength. A variety of treatments of the membrane were carried out in an attempt to characterize the membrane component(s) responsible for the binding of the Krebs cycle enzymes (Table III). Trypsin treatment decreased the binding somewhat (30%), but further treatment of the membranes with trypsin caused a dissolution of the membranes. Extraction of the membranes with 0.5 M KCl, which removes some membrane-associated proteins as shown by gel electrophoresis of this fraction (data not shown), or with toluene, which removes some membrane lipids (29), did not affect the binding capacity of the membranes. Acetic acid and urea are solvents which have been employed to remove extrinsic but firmly held inner membrane proteins (4, 30). Treatment of the membranes with these solvents markedly reduced the binding capacity of the membranes. However, it is possible that these solvents irreversibly denatured the binding sites instead of extracting them.

Finally, we were able to demonstrate the membrane specificity and orientation of the binding site in the inner mem-

**TABLE I**

| Enzyme                          | Source                      | Precipitate | Supernatant |
|---------------------------------|-----------------------------|-------------|-------------|
| Citrate synthase                | Pig heart                   | 3.6 ± 0.38  | 4.9 ± 0.4   | 42          |
| Malate dehydrogenase            | Pig heart (mitochondria)    | 67.6 ± 4.0  | 2.9 ± 0.7   | 96          |
| Malate dehydrogenase            | Pig heart (cytosol)         | 0.61 ± 0.09 | 33.8 ± 2.0  | 2           |
| Fumarase                        | Pig heart                   | 9.72 ± 0.82 | 13.0 ± 1.0  | 42          |
| Glucose-6-phosphate dehydrogenase| Yeast                      | 0.12 ± 0.1  | 7.6 ± 0.9   | 2           |
| Alcohol dehydrogenase           | Yeast                       | 0.5 ± 0.1   | 7.2 ± 0.5   | 6           |
| Citrate synthase                | Yeast                       | 0.33 ± 0.2  | 6.4 ± 0.1   | 5           |

**TABLE II**

Interaction of rat liver mitochondrial inner membranes with enzymes present in matrix and cytosolic fractions

Rat liver mitochondrial inner membrane (700 μg) and 1 mg of matrix (m) or cytosolic (c) proteins in 400 μl of 2 mM Hepes, pH 7.0, and 0.5 mM dithiothreitol were incubated at 0 °C for 15 min. The membranes were resuspended by centrifugation, washed, and enzyme activities were estimated as described in the text. The values represent mean ± S.E. of three experiments.

| Protein                          | Total enzyme activity bound | Enzyme activity bound | Activity bound |
|----------------------------------|-----------------------------|-----------------------|----------------|
| Matrix malate dehydrogenase      | 3.90 ± 0.3                  | 2.86 ± 0.3            | 73             |
| Cytosolic malate dehydrogenase   | 4.30 ± 0.4                  | 0.25 ± 0.2            | 6              |
| Matrix aspartate amino transferase| 3.20 ± 0.2                  | 2.75 ± 0.4            | 86             |
| Cytosolic aspartate amino transferase| 0.90 ± 0.1                  | 0.20 ± 0.04           | 22             |
| Matrix fumarase                  | 0.60 ± 0.05                 | 0.46 ± 0.04           | 77             |
| Cytosolic fumarase               | 0.30 ± 0.06                 | 0.02 ± 0.02           | 7              |
| Citrate synthase                 | 0.35 ± 0.05                 | 0.11 ± 0.05           | 31             |
| Matrix protein                   | 1065 μg ± 26                | 160 μg ± 20           | 15             |
| Cytosolic protein                | 1069 μg ± 32                | 40 μg ± 7             | 4              |
Binding of Citrate Synthase to Mitochondrial Inner Membranes

![Graph showing the effect of KCl on the binding of citrate synthase and malate dehydrogenase to rat liver mitochondrial inner membranes.](image1)

![Graph showing the effect of pH on citrate synthase binding to rat liver mitochondrial inner membranes.](image2)

![Graph showing specific binding of citrate synthase to rat liver mitochondrial inside out inner membranes.](image3)

**Table III**

| Treatment | Citrate synthase bound | Inhibition |
|-----------|------------------------|------------|
| None      | 3.1 ± 0.28             |            |
| Trypsin*  | 2.0 ± 0.12             | 36         |
| 7% Acetic acid* | 1.00 ± 0.09  | 88         |
| 4 M Urea* | 1.50 ± 0.1             | 52         |
| 0.2 M KCl* | 3.1 ± 0.2             | 0          |
| 0.5 M KCl* | 2.8 ± 0.2             | 10         |
| 2% Toluene* | 3.1 ± 0.32            | 0          |

* Rat liver mitochondrial inner membranes (3 mg) were incubated in 200 μl of 2 mM Hepes buffer, pH 7.0, containing 45 μg of trypsin for 10 min at room temperature. The proteolysis was stopped by the addition of trypsin inhibitor.

**Legend:**
- **Rat liver mitochondrial inner membranes (3 mg)** were incubated with either mitochondria (7.3 mg), mitoplast (2.4 mg), or inner membrane (700 μg) in a medium (400 μl) containing 220 mM mannitol, 70 mM sucrose, and 2 mM Hepes, pH 7.0, for 15 min at 0 °C. The mixture was centrifuged and washed, and citrate synthase activity in different preparations was estimated. In this experiment, the quantity of mitochondria used was chosen so that the total amount of outer membrane was about 700 μg to be equivalent with the quantity of inner membranes used. Similarly, the amount of mitoplasts used contained about 700 μg of inner membrane.

**Evidence for, and the metabolic implications of, enzyme binding to membranes:**

The evidence for, and the metabolic implications of, enzyme binding to membranes has been thoroughly reviewed (31). There are similarities between certain aspects of the binding of the Krebs cycle enzymes to the inner membrane and the binding of glycolytic enzymes to red blood cell membranes (32-34). In the latter case, sensitivity to high ionic strength is observed as well as competition between enzymes (32). Kliman and Steck (35) have pointed out that a consideration of mass action makes it likely that the poor binding of glyceroldehyde 3-phosphate to red cell ghosts at isotonic ionic strength is an

**Discussion:**

The evidence for, and the metabolic implications of, enzyme binding to membranes has been thoroughly reviewed (31). There are similarities between certain aspects of the binding of the Krebs cycle enzymes to the inner membrane and the binding of glycolytic enzymes to red blood cell membranes (32-34). In the latter case, sensitivity to high ionic strength is observed as well as competition between enzymes (32). Kliman and Steck (35) have pointed out that a consideration of mass action makes it likely that the poor binding of glyceroldehyde 3-phosphate to red cell ghosts at isotonic ionic strength is an
artifact of high dilution rather than due to a low affinity. It is
difficult to assess whether the results presented here have
physiological relevance. The binding conditions used here are
not normal. I have pointed out previously (11) that the high
protein content of the matrix resulted in an unusual set of
conditions in this compartment which may be relevant to a
putative structure of the tricarboxylic acid metabolic pathway.
In any case, we have observed a rather specific interaction
between soluble Krebs cycle enzymes and an oriented recep-
tor, probably a protein, in the inner membrane. We are
presently attempting to isolate the protein(s) responsible for
this binding.

Acknowledgment.—We would like to thank Reginald L. Tyiska for
his excellent technical assistance.

REFERENCES
1. Stanley, C. J., and Perham, R. N. (1980) Biochem. J. 191, 147–
154
2. Matlib, M. A., and O’Brien, P. J. (1975) Arch. Biochem. Biophys.
167, 193–202
3. Barnard, T., Azzelius, B. A., and Lindberg, D. (1971) J. Ultra-
struct. Res. 34, 544–566
4. Capaldi, R. A., and Tan, P.-F. (1974) Biochimie (Paris) 60,
1299–1305
5. Elduque, A., Casado, F., Cortes, A., and Bozal, J. (1982) Int. J.
Biochem. 14, 221–229
6. Wit-Peeters, E. M., Scholte, H. R., Van Den Akker, F., and De
Nie, I. (1971) Biochim. Biophys. Acta 231, 23–31
7. Scholte, H. R. (1969) Biochim. Biophys. Acta 178, 137–144
8. Wit-Peeters, E. M. (1969) Biochim. Biophys. Acta 178, 453–462
9. Landriscina, C., Papa, S., Comelli, P., Mazzarella, L., and Quag-
liariello, E. (1970) Biochim. Biophys. Acta 205, 136–147
10. Srere, P. A. (1980) Trends Biochem. Sci. 5, 120–121
11. Srere, P. A. (1982) Trends Biochem. Sci. 7, 375–378
12. Matlib, M. A., Shannon, W. A., and Srere, P. A. (1976) 34th
Annual Proceedings of the Electron Microscopy Society of
America Miami Beach, Florida (G. W. Bailey, ed)