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Intramitochondrial Localization and Proposed Metabolic Significance of Serine Transhydroxymethylase†

Raymond L. Cybulski and Ronald R. Fisher*

ABSTRACT: Serine transhydroxymethylase is a latent enzyme of intact rat liver mitochondria. The enzyme is neither solubilized by the selective removal of the outer membrane with digitonin, nor inactivated by concentrations of diazobenzenesulfonate that do not penetrate the inner membrane, but that do inhibit solubilized serine transhydroxymethylase. Swelling of mitochondria was studied in isoosmotic solutions of substrates under conditions that would define transport as neutral uniport, anion–hydroxyl exchange, anion–anion exchange, or electrophoretic. L-Serine and glycine appear to be rapidly taken up by a nonelectrophoretic uniport mechanism, while folate and tetrahydrofolate are not transported. The results localize the enzyme in the matrix and indicate that the latent activity results from a lack of tetrahydrofolate transport across the inner membrane. Based on these results, the dual localization of serine transhydroxymethylase in the mitochondria and the cytosol is proposed to provide a one-carbon shuttle system to link one-carbon metabolism in the two-cellular compartments.

Serine transhydroxymethylase (L-serine-tetrahydrofolate 5,10-hydroxymethyltransferase, EC 2.1.2.1), which catalyzes the reaction shown in eq 1, has been found to exist in approximately equal proportions as cytosolic and mitochondrial isozymes in rat liver (Nakano et al., 1968; Motokawa and Kikuchi, 1971; Palekar et al., 1973).

L-serine + *d*-L-tetrahydrofolate ⇌ glycine + *l*-L-5,10-methylenetetrahydrofolate + H_2O (1)

Yoshida and Kikuchi (1973) have suggested that serine transhydroxymethylase functions in the major pathway of serine catabolism in mammalian liver. The reaction may, therefore, be an important source of one-carbon units required for the biosynthesis of purines, thymidylate, and methionine. The isolation of a glycine-dependent mutant of Chinese hamster ovarian cells (Kao et al., 1969; Chasin et al., 1974) that is deficient in mitochondrial, but not cytosolic serine transhydroxymethylase, indicates the necessity of this isozyme for normal growth.

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In this communication, mitochondrial serine transhydroxymethylase is shown to exist as a soluble matrix enzyme by several localization techniques. Studies are also reported which suggest that L-serine and glycine are transported readily across the inner mitochondrial membrane, while an apparent lack of folate transport is demonstrated. Based on these results, a shuttle system functional in the transport of one-carbon units across the inner mitochondrial membrane and utilizing the two serine transhydroxymethylase isoenzymes is proposed.

Materials and Methods

Mitochondria were prepared from Sprague-Dawley rats, according to the procedure of Schnaitman and Greenawalt (1968). Protein determinations were performed by the biuret method (Jacobs et al., 1956). Tetrahydrofolate was prepared by catalytic hydrogenation of folic acid (Hatefi et al., 1960), and stored in sealed serum bottles in vacuo (Caldwell et al., 1973). Diazobenzenesulfonate was synthesized by the method of Dilley et al. (1972). Digitonin, pyridoxal 5′-phosphate, D- and L-serine, glycine, folic acid, dimedone, hexokinase, and isocitrate were obtained from Sigma Chemical Co. Lubrol WX was obtained from General Biochemicals. NAD^+ and $NADP^+$ were purchased from P.L. Biochemicals, Inc. and L-[3-^{14}C]-serine from Amersham/Searle.

A modification of the Schnaitman and Greenawalt (1968) procedure for the localization of mitochondrial enzymes was employed. Portions of mitochondria containing 25 mg of protein (≈100 mg/ml) were treated with an equal volume of 0.25 M sucrose containing various concentrations of digitonin and incubated for 15 min at 0 °C with gentle stirring. Three volumes of 0.25 M sucrose were then added and the samples were centrifuged for 10 min at 8500*g*. The supernatant solutions were gently removed and the pellets were resuspended in 1.5 ml of 0.25 M sucrose and again centrifuged for 10 min at 8500*g*. The supernatant solution was combined with the one previously obtained and the resultant solution was assayed for adenylate kinase (Schnaitman and Greenawalt, 1968), NADP-dependent isocitrate dehydrogenase (Plant and Sung, 1955), and serine transhydroxymethylase activities (Taylor and Weissbach, 1965). The inner membrane-matrix pellets were resuspended in 0.5 ml of 0.25 M sucrose containing 7.5 mg of lubrol and incubated for 15 min at 0 °C to disrupt the intact inner membranes. These extracts were diluted with 3.0 ml of 0.25 M sucrose and centrifuged for 30 min at 164 000*g*. The supernatant solution was assayed for adenylate kinase, NADP-dependent isocitrate dehydrogenase, and serine transhydroxymethylase activities.

Intramitochondrial enzyme localization studies, using diazobenzenesulfonate inactivation as a probe, were performed by preincubating 5 mg of mitochondrial protein in the dark for 30 min at 0 °C with various concentrations of diazobenzenesulfonate contained in 0.25 M sucrose (final volume, 1.0 ml). Reactions were terminated by addition of Tris-HCl (pH 7.4) to a final concentration of 200 mM. The mixtures were centrifuged for 10 min at 8500*g* and the supernatant solutions were discarded. The mitochondrial pellets were resuspended in 200 μl of 1.25% Lubrol and incubated for 15 min at 0 °C. This suspension of disrupted mitochondria was adjusted to 1.0 ml with 0.25 M sucrose and centrifuged for 30 min at 164 000*g*. Supernatant solutions were monitored for adenylate kinase, NADP-dependent isocitrate dehydrogenase, and serine transhydroxymethylase activities. In control experiments, mitochondria were disrupted by treatment with 0.5 mg of Lubrol/mg of protein for 15 min at 0 °C. Portions of this suspension containing 5 mg of protein were treated with diazobenzenesulfonate exactly as described above except that the 10-min 8500*g* centrifugation and following pellet resuspension were not performed.

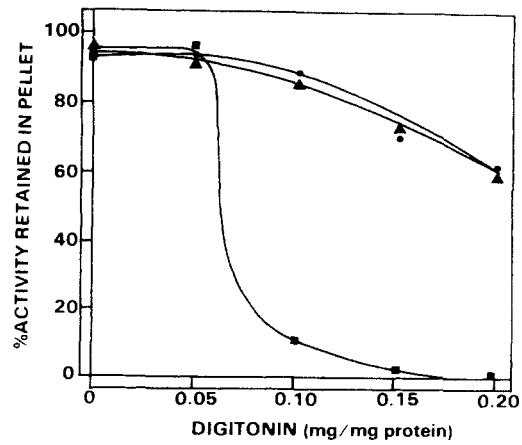

FIGURE 1: Effect of digitonin preincubation on the release of mitochondrial enzymes. Mitochondria were preincubated with various concentrations of digitonin, pelleted by centrifugation, disrupted by Lubrol treatment, and centrifuged as described under Materials and Methods. The supernatant solution obtained from this procedure was then monitored for adenylate kinase (■), NADP-dependent isocitrate dehydrogenase (▲), and serine transhydroxymethylase activities (●).

In latency experiments, a modified Taylor and Weissbach (1965) procedure was used to assay intact and Lubrol disrupted mitochondria for serine transhydroxymethylase activity. The final volume of the incubation mixture was 0.45 ml and contained 66.7 mM potassium phosphate (pH 7.4), 8.9 mM 2-mercaptoethanol, 0.22 mM pyridoxal 5′-phosphate, 1.78 mM tetrahydrofolate, 8.9 mM L-[3-^{14}C]serine (2×10^5 cpm), 250 mM sucrose, and 5.0 mg of mitochondrial protein. Reactions were started by the addition of protein and were incubated at 15 °C for the indicated time. Reaction termination and quantitation of methylenetetrahydrofolate were carried out according to the method of Taylor and Weissbach (1965). Mitochondrial intactness was monitored by measuring the rate of NADH oxidation uptake measured with a Gilson Model KN oxygraph equipped with a Ysi 4040 Clark oxygen probe.

Mitochondrial transport studies utilizing osmotic-swelling techniques were performed by the general method of Chappell and Haarhoff (1967). Mitochondria (1 mg of protein in ≈40 μl of 0.25 M sucrose) were suspended in a 200 mosM solute solution (pH 7.4) supplemented with 5 mM Tris-HCl (pH 7.4), 0.3 mM EDTA, and 5 mM KCN, such that the final volume was 1.0 ml. Mitochondrial volume changes were observed by monitoring the change in absorbance at 610 nm.

Results

The Schnaitman and Greenawalt (1968) procedure for intramitochondrial enzyme localization was utilized to determine the location of serine transhydroxymethylase. At a proper concentration of digitonin, the outer membranes are completely disrupted releasing intermembrane space enzymes, while the inner membranes remain intact. The inner membranes can then be isolated and ruptured by Lubrol treatment to release soluble matrix enzymes. Figure 1 shows the percentage of marker enzyme activity released from digitonin-

[1] Abbreviations used are: NAD, nicotinamide adenine dinucleotide; NADH, reduced NAD; NADP, NAD phosphate; EDTA, (ethylenedinitrilo)tetraacetic acid.

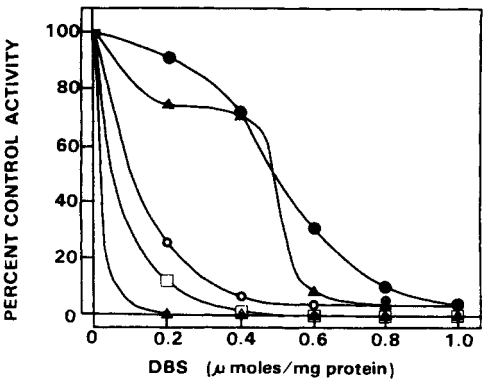

FIGURE 2: Diazobenzenesulfonate inhibition as a probe in mitochondrial enzyme localization. Intact mitochondria were preincubated with various concentrations of diazobenzenesulfonate, pelleted by centrifugation, disrupted by Lubrol treatment, and centrifuged to form pellet and supernatant solution fractions, as described under Materials and Methods. The supernatant solution was then monitored for adenylate kinase (■), NADP-dependent isocitrate dehydrogenase (▲), and serine transhydroxymethylase (●) activities. Control experiments preincubating disrupted mitochondria with various concentrations of diazobenzenesulfonate, as described under Materials and Methods, were also performed. The supernatant solutions obtained from these experiments were assayed for adenylate kinase (□), NADP-dependent isocitrate dehydrogenase (△), and serine transhydroxymethylase (○) activities.

pretreated mitochondria by Lubrol treatment against the amount of digitonin in the preincubation mixture. The release of adenylate kinase, an intermembrane marker, indicates that the outer membranes are almost completely disrupted between 0.05 and 0.10 mg of digitonin/mg of protein, while the release of only a small percentage of the matrix marker, NADP-dependent isocitrate dehydrogenase, shows that the inner membranes are not substantially affected by these digitonin concentrations. The similarity of the serine transhydroxymethylase and isocitrate dehydrogenase curves clearly illustrates that serine transhydroxymethylase is not a soluble intermembrane enzyme. Based on these results a more conclusive location of enzyme cannot be defined, i.e., the enzyme could be bound to the outer or inner surface of the inner membrane and solubilized during Lubrol extraction, although it has generally been accepted that the release of enzyme activity from the inner membrane-matrix fraction by Lubrol treatment demonstrates a soluble matrix positioning of the enzyme (Schnaitman and Greenawalt, 1968).

Tinberg et al. (1974) demonstrated the use of diazobenzenesulfonate, an inner membrane impermeable enzyme inhibitor, in studying the asymmetric binding of enzymes to the mitochondrial inner membrane. In order to determine whether serine transhydroxymethylase is localized on the inner or outer side of the inner membrane barrier, intact mitochondria were pretreated with diazobenzenesulfonate and monitored for enzyme inactivation. Figure 2 represents the percentage of enzyme activity released from mitochondria by Lubrol treatment following preincubation with various diazobenzenesulfonate concentrations. Control experiments using ruptured instead of intact mitochondria demonstrate the susceptibility of adenylate kinase, NADP-dependent isocitrate dehydrogenase, and serine transhydroxymethylase to diazobenzenesulfonate inactivation. The drastic decrease in adenylate kinase activity upon treatment of intact mitochondria with low concentrations of this reagent indicates that it is penetrating the outer membrane and inhibiting the intermembrane-space enzyme. At this same inhibitor concentration, however, the amount of isocitrate dehydrogenase activity remaining in intact mitochondria is substantially greater than that observed in the disrupted mitochondrial control, thus demonstrating that the inner membrane acts as a permeability barrier between the probe and the matrix enzyme. The retention of serine transhydroxymethylase activity in intact mitochondria at these lower diazobenzenesulfonate concentrations shows that this enzyme is also being protected by the inner membrane permeability barrier. As can be seen in Figure 2, both serine transhydroxymethylase and isocitrate dehydrogenase are inhibited at higher diazobenzenesulfonate concentrations. Thus, the reagent appears to permeate the inner membrane under these conditions. Clearly, caution must be exercised in using this probe for studying enzyme localization. Isocitrate dehydrogenase, as shown by the disrupted mitochondria control, is very susceptible to diazobenzenesulfonate inactivation. The biphasic nature of the isocitrate dehydrogenase inhibition curve in intact mitochondria may, therefore, be caused by the existence of two mitochondrial populations of differing inhibitor permeability.

Although the previous results are consistent with a soluble matrix location for serine transhydroxymethylase, the possibility of the enzyme being bound to the inner side of the inner membrane and released by detergent treatment cannot be neglected. In order to check this possibility, serine transhydroxymethylase assays were performed on soluble and inner-membrane fractions obtained from mitochondria disrupted by sonication. Results of such experiments show that over 91% of the total serine transhydroxymethylase activity is found in the soluble fraction, thus supporting the notion that the enzyme exists in a soluble form in the matrix.

Another indication that serine transhydroxymethylase exists as a matrix enzyme is its latency. The apparent specific activity of serine transhydroxymethylase of Lubrol-disrupted mitochondria is approximately tenfold higher than that of intact mitochondria. This indicates that the enzyme is in the matrix and that either L-serine or tetrahydrofolate is not transported across the inner membrane.

Chappell et al. (1972) have reported three apparent mechanisms for anion transport across the inner mitochondrial membrane: (1) anion-hydroxyl exchange, (2) anion-anion exchange, and (3) electrophoretic transport. Mitochondrial-swelling techniques similar to those utilized to elucidate these three mechanisms were applied to study the transport of serine transhydroxymethylase substrates. Anion-hydroxyl exchange is indicated by swelling in the presence of either isoosmotic ammonium salts of a transported anion or with its potassium salt in the presence of valinomycin and a classical oxidative phosphorylation uncoupler. Electrophoretic transport of ammonium or potassium salts of anions occurs only in the presence of uncoupler or valinomycin, respectively, while in anion-anion exchange systems transport is observed only in the presence of the appropriate counterion. Little if any swelling of mitochondria occurred in the presence of either the ammonium or potassium salts of folic acid or tetrahydrofolate. Similar results were obtained when these experiments were performed in the presence of potential exchange transport substances (glycine, L-serine, malate, phosphate, or succinate), either in the presence or absence of uncoupler (carbonyl cyanide *m*-chlorophenylhydrazone), valinomycin, or both. Figure 3 shows representative results obtained with the ammonium salts of folic acid and tetrahydrofolate. A lack of folate transport across the inner membrane is suggested by these results, but the existence of an unknown anion-anion exchange system involving folate coenzymes cannot yet be disproven.

Other conceivable explanations for the lack of tetrahydro-

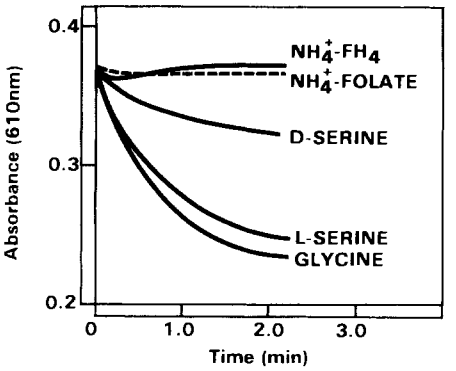

FIGURE 3: Mitochondrial swelling in the presence of folic acid, tetrahydrofolate, D-serine, L-serine, and glycine. Tetrahydrofolic acid and folic acid were adjusted to pH 7.4 with ammonium hydroxide, while L-serine, D-serine, and glycine were adjusted to pH 7.4 with sodium hydroxide. Mitochondria were added to an isoosmolar solution of the indicated compound and swelling was monitored as described under Materials and Methods.

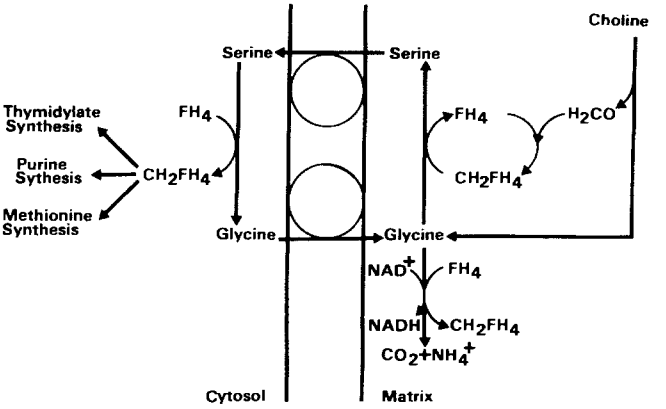

FIGURE 4: Proposed one-carbon unit transport shuttle.

folate transport derive from the facts that (a) the transport of a racemic mixture of tetrahydrofolate was studied and (b) a significant fraction of the total folate pool consists of various folylpolyglutamates and other folylmonoglutamates (Wang et al., 1967; Osborne-White and Smith, 1973). If a carrier for tetrahydrofolate exists, then the unnatural (*l*-L) isomer might act as a transport inhibitor of the natural (*d*-L) isomer. It is also possible that carriers not functional with tetrahydrofolate may facilitate the transport of other folate coenzymes.

Mitochondria demonstrate rapid swelling in the presence of either glycine or L-serine at pH 7.4. These results agree with a report by Gamble and Lehninger (1973), in which glycine and certain other neutral amino acids (alanine, valine, and proline) were shown to readily permeate the mitochondrial inner membrane. At physiological pH both serine and glycine exist predominately (>98%) as zwitterions and their uptake by mitochondria appears independent of ammonium or potassium ions plus valinomycin. It is, therefore, apparent that the transport of glycine and L-serine does not occur by an anion–anion or anion–hydroxyl exchange, nor is it an electrogenic process. These compounds most likely enter the mitochondrial matrix as neutral species. Unlike glycine and L-serine, D-serine induced only a slow, lower magnitude swelling.

Discussion

Several reports suggest that folates are associated with mitochondria, but no intramitochondrial localization of the coenzymes has been performed (Gawthorne and Smith, 1973; Wang et al., 1967; Frisell and Randolph, 1973). The present serine transhydroxymethylase localization verifies the existence of a pool of reduced folate coenzymes in the mitochondrial matrix. However, the folic acid and tetrahydrofolate transport studies indicate a lack of folate transport across the mitochondrial inner membrane, and suggest the existence of separate cytosolic and mitochondrial folate coenzyme pools reminiscent of the nicotinamide dinucleotide pools previously proposed by Greenspan and Purvis (1968). The existence of such pools raises questions concerning the mode of replenishment of matrix coenzymes. It has been shown, in the case of nicotinamide dinucleotides, that a very slow equilibration of cytosolic and mitochondrial pools occurs. Similarly, certain folate coenzymes may transverse the inner membrane so slowly as to make this process per se metabolically insignificant. Glycine and L-serine, on the other hand, appear to be readily transported across the mitochondrial inner membrane as their zwitterions. Furthermore, the existence of a transport carrier, at least for L-serine, is implied by the substantial difference between the rates of D- and L-serine transport.

Cytoplasmic and mitochondrial isozymes of malate dehydrogenase and glutamate-oxalacetate transaminase have been implicated in the transfer of reducing equivalents across the mitochondrial inner membrane (Borst, 1963). Since serine transhydroxymethylase isozymes catalyze the transfer of a one-carbon unit, it is conceivable that they are involved in the transport of one-carbon units across the inner membrane. Such a proposed shuttle is shown in Figure 4. Formaldehyde formed during intramitochondrial choline catabolism at the level of sarcosine and dimethylglycine oxidation reacts nonenzymatically with tetrahydrofolate to form methylenetetrahydrofolate (Blakley, 1963). Mitochondrial serine transhydroxymethylase then transfers the hydroxymethyl group to glycine to form serine. The formation of ^{14}C-labeled serine from dimethylglycine and sarcosine containing ^{14}C-labeled *N*-methyl groups has previously been observed by McKenzie and Frisell (1958). The intramitochondrial serine is subsequently transported to the cytosol where it is reconverted to glycine by cytosolic serine transhydroxymethylase. Methylenetetrahydrofolate produced during this reaction serves as the one-carbon source necessary for certain major biosyntheses, while the glycine regenerated during the reaction may then be transported back into the matrix to complete the cycle. Alternatively, intramitochondrial glycine may also be catabolized by a glycine cleavage system to form NH_3, CO_2, NADH, and methylenetetrahydrofolate (Kikuchi, 1973). The glycine cleavage system, although shown in Figure 4 as being located in the matrix, has not been localized conclusively and may be bound to the outer side of the inner membrane. The methylenetetrahydrofolate so formed could then enter the one-carbon unit shuttle system.

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Amyloid A: Amphipathic Helixes and Lipid Binding†

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ABSTRACT: Polypeptide segments, composed of α helixes with specific surface topography termed amphipathic helixes, have been proposed as the basic lipid-associating domains of apolipoproteins from the plasma lipoproteins. A computer search for proteins having sequences that could form amphipathic helixes indicated that amyloid A, a pathologically occurring protein usually associated with "secondary" amyloidosis, also contained amphipathic helixes. In studies reported here, amyloid A is shown to associate spontaneously with phospholipid vesicles with the following results: (a) the formation of a protein–lipid complex isolated by equilibrium density gradient ultracentrifugation, (b) a 100% increase in α helicity as measured by circular dichroism, (c) a 9-nm shift in the fluorescence maximum due to the single tryptophan residue located in the amphipathic region, indicating the tryptophan is moving from a polar to a nonpolar environment, and (d) the formation of stacked disk-like protein–lipid complexes as visualized by negative stain electron microscopy. The temperature dependence of the circular dichroic spectrum of the amyloid A–phospholipid complex suggests that the complex is formed by insertion of protein between the fatty acyl chains of the lipid. These findings suggest that the amphipathic helix is an important structural unit in lipid-associating proteins and that this unit can be recognized on the basis of its amino acid sequence. In addition, these studies have implications for the origin and function of amyloid A protein.

An α helix with a specific surface topography consisting of opposing polar and nonpolar faces and a regular charge distribution (termed an amphipathic helix) has been proposed as a basic structural element of the lipid-associating domains of apolipoproteins in very low-density lipoproteins (VLDL)[1] and high density lipoproteins (HDL) (Segrest et al., 1974). Potential amphipathic helixes (to be referred to as amphipathic sequences) can be identified from amino acid sequence data. Using a computer program based on this model, amphipathic sequences have been identified from known amino acid sequence data (Segrest, J. P., and Feldmann, R. J., submitted for publication).

The computer search for amphipathic sequences identified two amphipathic sequences from the amino acid sequence of amyloid A. As one test for the amphipathic helix model for protein–lipid association, amyloid A was examined for lipid-associative properties.

Amyloid A (Glenner et al., 1973; Ein et al., 1972; Benditt

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‡ Established Investigator of the American Heart Association.

[1] Abbreviations used are: VLDL, very low-density lipoprotein; HDL, high-density lipoprotein; DMPC, dimyristoylphosphatidylcholine; CD, circular dichroism; T_c, liquid crystalline phase transition temperature; EDTA, (ethylenedinitrilo)tetraacetic acid.