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Characterization of the Inhibitor Sensitivity of the Coenzyme A Transport System in Isolated Rat Heart Mitochondria

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The effect of protein labeling agents on coenzyme A (CoA) transport into isolated rat heart mitochondria was studied. CoA transport was substantially inhibited by sulfhydryl reagents (mersalyl, pCMB) as well as by the tyrosine-selective reagent N-acetylimidazole. The effect of pCMB was reversed by DTT. Moreover, CoA uptake was completely abolished by agents selective for lysine and amino terminal residues (pyridoxal 5-phosphate, dansyl chloride). In contrast arginine-selective reagents (2, 3-butanedione, phenylglyoxal) caused considerably less inhibiton of CoA uptake. Moreover, partial inhibition of transport was observed with the stilbene disulfonic acid derivatives DIDS and SITS. Finally, measurement of the effects of the labeling agents on the mitochondrial membrane potential indicated that the inhibiton of CoA transport into mitochondria is not a secondary effect that arises from an alteration in the electric potential gradient across the inner mitochondrial membrane. These results provide the first information on the types of amino acid residues that may be essential to the CoA transport mechanism and provide additional support for the existence of a CoA transport protein within the mitochondrial inner membrane. Furthermore, the identification of effective inhibitors of the CoA transport system will greatly facilitate the functional reconstitution of this transporter in a proteoliposomal system following its solubilization and purification.

KEY WORDS: Coenzyme A, mitochondria, transport, rat heart, protein labeling agents

INTRODUCTION

Cellular acylation reactions are mediated by CoA,[4] a cofactor which is synthesized in the cytosol of myocardial cells from the vitamin pantothenic acid (Tahiliani and Neely, 1987a). Following synthesis, CoA is transported into mitochondria. We have recently shown that CoA transport into mitochondria can be saturated (Tahiliani and Neely, 1987b), proceeds against a concentration gradient (Tahiliani and Neely, 1987b), and demonstrates dependence on the membrane potential (Tahiliani, 1989), thus suggesting that uptake may be catalyzed by a transport protein. If such a protein were involved, one would expect that transport would be affected by various protein-modifying reagents.

The present investigation was undertaken to determine the sensitivity of mitochondrial CoA transport to a variety of protein-modifying reagents which are selective for specific types of amino acid residues. Accordingly, we have characterized the sensitivity of this transport system to cysteine-, lysine-, arginine-, and tyrosine-selective reagents. This study provides the first information on the types of amino acids that may be essential to the CoA transport mechanism and

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[4]Abbreviations used: CoA, coenzyme A; dansyl chloride, 5-dimethylaminonaphthalene-1-sulfonyl chloride; DIDS, 4,4′-diisothiocyanato-stilbene-2,2′-disulfonic acid; pCMB, *p*-chloromercuribenzoic acid; NEM, *N*-ethylmaleimide; SE, standard error of the mean; SITS, 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid; TPP, tetraphenylphosphonium bromide.

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provides important tools for future studies focusing on functional reconstitution of the CoA transporter following solubilization and purification.

MATERIALS AND METHODS

Chemicals

For these studies all inhibitors were obtained from Sigma. [^{14}C]sorbitol was purchased from ICN Biomedicals, [1-^{14}C]pantothenic acid from NEN Research Products, and [^{3}H]TPP from Amersham.

Isolation of Mitochondria and Measurement of CoA Transport

Rat heart mitochondria were isolated by a modification of the method of Chance and Hagihara (1960) as described by Idell-Wenger *et al.* (1982). CoA uptake into mitochondria was studied in the presence of 10mM succinate as substrate as described previously (Tahiliani and Neely, 1987b; Tahiliani, 1989). Briefly, mitochondria were preincubated at 30°C with the protein-labeling agent under investigation as follows. For pyridoxal phosphate, mitochondria were preincubated with the reagent for 15 min after which $NaBH_4$ (15 mM final concentration) was added and the preincubation was carried out for an additional 5 min. Preincubations with 2,3-butanedione and dansyl chloride were carried out for 20 min. All other labeling agents were preincubated with mitochondria for 5 min. It should be noted that dansyl chloride was dissolved in absolute ethanol and all other labeling agents were dissolved in water. Also, with DIDS and SITS, the preincubations were carried out in the dark. In all cases, control incubations were carried out in the absence of protein-labeling agent. Finally, when the effect of sulfhydryl reagents was studied, DTT was omitted from the incubation medium and CoA was made up in distilled water instead of 10 mM DTT. This omission did not affect CoA levels in the incubation medium. When the ability of DTT to reverse the effect of pCMB on CoA uptake was studied, mitochondria were incubated with pCMB on ice for 5 min. Dithiothreitol (final concentration of 10 mM) was then added and the incubation continued at 30°C for an additional 5 min. CoA uptake was then carried out as described below.

CoA uptake was then initiated by the addition of 100 μM [^{14}C]CoA (specific radioactivity approximately 50 nCi/nmol prepared as previously detailed by Tahiliani, 1989) and aliquots were subsequently removed at 0.5 min and 25 min following the initiation of CoA transport and gently transferred to 1.5-ml Eppendorf tubes containing 300 μl silicone oil (an 83:17 mixture of 550 and 200 weight Dow Corning oils) and 200 μl of 14% perchloric acid and then centrifuged as previously described (Tahiliani and Neely, 1987b; Tahiliani, 1989). Parallel incubations were carried out with [^{14}C]sorbitol in order to determine the extramatrix space. Radioactivity in aliquots of the supernatant and the pellet was determined by liquid scintillation counting and the amount of CoA associated with mitochondria was then calculated from the specific activity of CoA in the supernatant. CoA uptake was then calculated as the increase in the amount of CoA associated with mitochondria between 0.5 and 25 min of incubation. The data reported in all tables were obtained from at least three separate mitochondrial preparations.

Miscellaneous Procedures

In order to ascertain whether or not the sulfhydryl reagents affected the CoA content of the incubation medium, CoA levels were determined in the supernatants of the Eppendorf tubes containing the 25-min post-incubation samples by the α-ketoglutarate dehydrogenase assay (Garland *et al.*, 1965). The electrical gradient across the mitochondrial inner membrane was determined in both the presence and absence of the protein-labeling agents by measuring the distribution of [^{3}H]TPP as described elsewhere (Tahiliani, 1989). For these measurements, the preincubation with a given agent was carried out for the time intervals described above. Moreover, the 0.5-min and 25-min points refer to the time elapsed following the simultaneous addition of CoA + [^{3}H]TPP after the preincubation. Protein was determined by the Biorad procedure (Bradford, 1976).

RESULTS AND DISCUSSION

Effect of Sulfhydryl Reagents on CoA Transport into Mitochondria

Initial experiments focused on determining whether sulfhydryl reagents were capable of inhibiting CoA transport into mitochondria. As shown in Table I, both mersalyl and pCMB substantially inhibited the CoA uptake system. In order to determine whether the observed inhibition of CoA transport was due to a

Table I. Effect of Sulfhydryl Reagents on Mitochondrial CoA Transport and on CoA Levels in Post-Incubation Supernatants[a]

Agent	Concentration (μM)	Inhibition of CoA transport (%)	CoA level (% of control)
Mersalyl	100	56 ± 7 (4)	95 ± 3 (4)
	200	78 ± 9 (4)	84 ± 4 (4)
	250	92 ± 6 (4)	58 ± 17 (4)
pCMB	100	74 ± 10 (6)	95 ± 2 (3)

[a] Mitochondria were preincubated with a given sulfhydryl reagent at 30°C for 5 min prior to the addition of [^{14}C]CoA as described in detail in Materials and Methods. In separate experiments, CoA levels were determined in the post-incubation supernatant in order to ascertain whether the sulfhydryl reagents altered the concentration of CoA in the incubation mix. Data represent means ± SE. Values in parentheses represent the total number of incubations. An average of all the experiments described in this table yielded a mean control rate of CoA uptake of 1.25 ± 0.11 nmol/24.5 min/mg protein (n = 9) and a mean concentration of CoA in the control of 74.9 ± 5.3 nmol/ml supernatant (n = 7).

Table II. Reversal of pCMB-Induced Inhibition of CoA Transport by DTT[a]

Condition	CoA transport (nmol/24.5 min/mg protein)	Inhibition (%)
Control (minus DTT)	0.87 ± 0.04	—
pCMB (minus DTT)	0.33 ± 0.07	62 ± 6
Control (plus DTT)	0.80 ± 0.07	—
pCMB (plus DTT)	0.83 ± 0.06	−4 ± 8

[a] Mitochondria were preincubated with either 100 μM pCMB or water (control) for 5 min on ice prior to the addition of 10 mM DTT. Following a 5-min equilibration at 30°C, 100 μM [^{14}C]CoA was added to initiate transport. Data represent means of three experiments ± SE.

decrease (by the sulfhydryl reagent) in the amount of CoA present in the transport reaction mix instead of a direct effect on the CoA transport system, CoA levels were assayed (in a separate set of experiments) immediately following the transport incubation. As shown in Table I, considerable inhibition of CoA transport was observed at sulfhydryl reagent concentrations which did not cause substantial changes in the level of CoA. Thus, these findings rule out the possibility that the observed inhibition of transport arose due to a chemical alteration of the transport substrate. This conclusion is further supported by the fact that the concentration of CoA utilized in these incubations was approximately 5-fold higher than the K_m for CoA transport (Tahiliani and Neely, 1987b) and thus small variations in CoA levels would not significantly alter the CoA transport rate. It is also of interest to note that as indicated in Table II, the effect of pCMB was reversed by the addition of 10 mM DTT. This observation permits the conclusion that pCMB inhibition of CoA transport was mediated via interaction with protein sulfhydryl group(s). The ability of sulfhydryl reagents to inhibit CoA transport into mitochondria suggests that cysteine residue(s) are essential to the transport mechanism. Furthermore, the observation that these agents exert similar inhibitory effects on other mitochondrial anion transporters (LaNoue and Schoolwerth, 1979; Bryla, 1980) suggests a mechanism for mitochondrial anion transport that may involve similar structural domains among the different anion carriers. This idea has recently been proposed (Aquila *et al.*, 1987; Runswick *et al.*, 1987) based on amino acid sequence homology between several mitochondrial transporters (Aquila *et al.*, 1982, 1985, 1987; Runswick *et al.*, 1987; Ferreira *et al.*, 1989).

In contrast to the results that we obtained with higher concentrations of labeling agent (Table I), low concentrations of mersalyl (25 μM) and pCMB (10 μM) caused a significant stimulation of CoA uptake (i.e., 40 and 70% increases in CoA uptake were observed, respectively; data not shown). While the mechanism of this stimulation is unclear at present, it is tempting to speculate that it may arise as a consequence of an increased matrix pH caused by these agents. This hypothesis is supported by earlier findings that mercurials enhance both proton ejection and monovalent cation/H^+ exchange across the mitochondrial inner membrane (Southard *et al.*, 1973; Southard *et al.*, 1974) and that alkalinization of the mitochondrial matrix enhances the rate of CoA transport (Tahiliani, 1989). In the present study, the enhancement of CoA transport at low concentrations of mercurials is reversed to an inhibition of transport at higher concentrations, presumably due to an interaction of protein labeling agent with thiol groups present within the CoA transport system. Finally, it should be noted that although experiments were conducted employing the sulfhydryl reagent NEM, considerable chemical alteration of the CoA present in the incubation mix was observed even at relatively low concentrations of this agent (i.e., 33% and 58% depletion of CoA levels at 25 and 50 μM NEM, respectively). Thus an accurate assessment of the effect of NEM on the CoA transport system could not be made.

Table III. Effect of Lysine- and Arginine-Selective Reagents on Mitochondrial CoA Transport[a]

Agent	Concentration (mM)	Inhibition (%)
Pyridoxal 5-phosphate	10	105 ± 4 (3)
Dansyl chloride	3	105 ± 8 (3)
2,3-Butanedione	10	13 ± 11 (3)
Phenylglyoxal	10	13 ± 11 (5)
	20	38 ± 7 (3)

[a] Mitochondria were preincubated with a given agent at 30°C prior to the addition of [^{14}C]CoA as described in detail in Materials and Methods. Data represent mean ± SE. Values in parentheses represent the total number of incubations performed. The average of all experiments described in this table yielded a mean control CoA uptake rate of 1.03 ± 0.09 nmol/24.5 min/mg protein (15).

Table IV. Effect of Other Types of Protein-Labeling Reagents on Mitochondrial CoA Transport[a]

Agent	Concentration (mM)	Inhibition (%)
N-Acetylimidazole	12.5	57 ± 5 (4)
	25.0	66 ± 11 (3)
DIDS	0.01	4 ± 9 (4)
	0.05	39 ± 10 (4)
SITS	0.50	40 ± 3 (4)

[a] Mitochondria were preincubated with a given agent at 30°C for a total of 5 min prior to the addition of [^{14}C]CoA as described in detail in Materials and Methods. Data represent means ± SE. Values in parentheses represent the total number of incubations performed. The average of all experiments described in this table yielded a mean control CoA uptake rate of 0.97 ± 0.08 nmol/24.5 min/mg protein (18).

Effect of Lysine- and Arginine-Selective Labeling Agents on CoA Transport into Mitochondria

We then examined the effect of lysine- and arginine-selective reagents (Lundblad and Noyes, 1984) on CoA transport into isolated mitochondria. As depicted in Table III, the reagents pyridoxal 5-phosphate and dansyl chloride, which are specific for lysine as well as amino terminal residues, completely abolished the CoA uptake process. In contrast, the arginine-selective reagents 2,3-butanedione and phenylglyoxal caused considerably less inhibition of CoA transport. Thus, the results presented in Table III suggest that the CoA transport system contains a lysine and/or an amino terminal residue(s) that is both accessible to covalent modifying agents and essential to the translocation mechanism. In contrast, arginine residues within the transporter appear to be either considerably less accessible and/or less important in the mechanism of transport. Finally, it is interesting to note that the CoA transport system displays a sensitivity to lysine-selective agents which is similar to that displayed by other mitochondrial transporters, but displays a reduced sensitivity to arginine-selective agents (Kramer, 1984; Kaplan *et al.*, 1986, 1990; Hutson *et al.*, 1990). However, these comparisons are rather tentative since, in contrast to the present study, which was carried out with isolated mitochondria, the investigations of the inhibitor sensitivities of other anion transporters were carried out with purified transporters that had been functionally reconstituted in proteoliposomal systems. Clearly the accessibility of a given amino acid to added reagent may vary depending upon the lipid environment in which the transporter of interest is situated.

Effect of Other Protein-Labeling Agents on the CoA Transport System

Table IV depicts the effect of other covalent modifying reagents on CoA transporter function. Interestingly, *N*-acetylimidazole, a tyrosine-selective reagent, effectively inhibited CoA transport. In this respect, the CoA transporter displays a sensitivity similar to that observed with other mitochondrial anion carriers (Kaplan *et al.*, 1986; Hutson *et al.*, 1990). Additionally, employing concentrations of the stilbene disulfonic acid derivatives DIDS and SITS, which inhibit other anion-binding proteins (Cabantchik and Rothstein, 1972, 1974; Cabantchik *et al.* 1978; Zoccoli and Karnovsky, 1980; Claeys and Azzi, 1989), we observed a partial inhibition of mitochondrial CoA transport, thereby providing further support for the participation of a lysine (and/or the amino terminal) residue(s) in the transport mechanism.

In a final series of experiments, we examined the effects of the above protein-labeling reagents on the electrical gradient across the mitochondrial inner membrane since these agents can affect the membrane electrical gradient (Marzulli and Lofrumento, 1985; Zanotti *et al.*, 1985) and since we have previously reported that CoA uptake is driven by the membrane electrical gradient (Tahiliani, 1989). As depicted in Table V, mersalyl, pCMB, pyridoxal 5-phosphate, dansyl chloride, and phenylglyoxal caused a moderate to substantial decrease in the electric potential across the mitochondrial inner membrane (i.e., 33–65% decreases were observed; values refer to data obtained after preincubation with a given labeling agent plus a subsequent 25-min incubation with CoA + TPP). In contrast, *N*-acetylimidazole did not alter the electric

Table V. Effect of Protein-Labeling Reagents on the Electrical Gradient across the Mitochondrial Inner Membrane[a]

Agent	Electrical gradient across the inner membrane			
	0.5 min		25 min	
	(mV)	(Percent Decrease)	(mV)	(Percent Decrease)
Control	−118.5 ± 3.8	—	−92.0 ± 1.4	—
Mersalyl (100 μM)	−72.8 ± 9.4	39	−32.1 ± 2.7	65
(200 μM)	−84.0 ± 12.9	29	−36.3 ± 1.9	61
(250 μM)	−66.1 ± 18.1	44	−33.0 ± 7.4	64
pCMB (100 μM)	−92.3 ± 5.2	22	−36.8 ± 2.5	60
Control	−118.2 ± 3.3	—	−84.1 ± 1.1	—
Pyridoxal 5-phosphate (10 mM)	−65.0 ± 15.0	45	−50.7 ± 8.5	40
Dansyl Chloride (3 mM)	−74.2 ± 3.6	37	−56.7 ± 2.6	33
Phenylglyoxal (20 mM)	−78.4 ± 7.9	34	−54.5 ± 1.3	35
N-acetylimidazole (12.5 mM)	−118.4 ± 5.0	0	−82.8 ± 2.0	2
(25 mM)	−123.5 ± 2.9	−4	−83.8 ± 1.5	0
DIDS (50 μM)	−123.3 ± 3.5	−4	−108.1 ± 2.0	−29
SITS (0.5 mM)	−121.8 ± 2.3	−3	−105.9 ± 5.8	−26

[a]Data represent means (of four experiments) ± SE. The preincubation of mitochondria with a given labeling agent and the subsequent measure of the electrical gradient across the mitochondrial inner membrane were performed as described in Materials and Methods.

potential, and DIDS and SITS actually caused an increase in potential. Table VI depicts a direct comparison of the effect of a given labeling agent on membrane potential with its effect on CoA transport into mitochondria. The agents have been divided into three classes based upon the extent to which they altered the membrane potential. The Class I agents caused the largest decrease in the electric potential

Table VI. Comparison of the Effect of Protein-Labeling Reagents on Both the Membrane Potential and CoA Transport[a]

Agent	Concentration	Change in mitochondrial membrane potential (Percent Decrease)	Change in CoA Transport (Percent Inhibition)
Class I Agents			
Mersalyl	100 μM	65	56
Mersalyl	200 μM	61	78
Mersalyl	250 μM	64	92
pCMB	100 μM	60	74
Class II Agents			
Pyridoxal 5-phosphate	10 mM	40	105
Dansyl chloride	3 mM	33	105
Phenylglyoxal	20 mM	35	38
Class III Agents			
N-acetylimidazole	12.5 mM	2	57
N-acetylimidazole	25 mM	0	66
DIDS	0.05 mM	−29	39
SITS	0.5 mM	−26	40

[a]Data were obtained from Tables I–V. Class I Agents, caused the largest decrease (i.e., 60–65%, 25 min following the CoA + [^{3}H]TPP addition) in the electric potential across the mitochondrial inner membrane. Class II Agents caused a less severe decrease (i.e., 33–40%) in membrane potential. Class III Agents had either little effect or caused an increase in the membrane potential (i.e., 2% decrease to 29% increase).

(60–65%) and resulted in a large inhibition of CoA transport (i.e., 56–92%). The Class II agents caused a less severe decrease in the membrane potential (i.e., 33–40%) but nonetheless caused a substantial inhibition of CoA transport (i.e., 38–105%). Finally, the Class III agents had either little effect on the electric potential or actually increased the potential (i.e., 2% decrease to 29% increase were observed), and also caused substantial inhibition of CoA transport (i.e., 39–66%). Taken together, these data permit the conclusion that a correlation does not exist between the ability of a given labeling agent to alter the membrane potential and its ability to inhibit CoA transport. Furthermore, in a separate series of experiments, we have recently discovered that the hemicalcium salt of pantothenic acid, which is the precursor for CoA, completely abolishes the electrical gradient across the mitochondrial inner membrane, yet it stimulates CoA uptake (unpublished observations). Thus, our previous hypothesis (Tahiliani, 1989) stating that CoA uptake is driven by the electrical gradient requires revision.

To summarize, the studies described in this communication permit the following conclusions. First, our present observation that certain types of protein-labeling agents inhibit CoA transport into mitochondria in combination with our previous findings (Tahiliani and Neely, 1987b), strongly suggest that CoA uptake into mitochondria is mediated by a transport protein. Second, our finding that the inhibitor sensitivity of CoA transport is similar but not identical to that observed for other mitochondrial anion transport proteins suggests that the CoA transporter is likely to contain similar as well as distinct structural domains that participate in the translocation mechanism. Along these lines, our results indicate that cysteine, lysine, and tyrosine residues may play essential roles in the transport process. However, it is important to note that firm conclusions concerning the actual residues involved in the translocation mechanism will require considerable further investigation. Third, the inhibition of CoA transport mediated by these agents appears to be a direct effect on the CoA transport system and is not a secondary effect that arises from an alteration in the electric potential gradient across the mitochondrial inner membrane. Finally, the identification of several protein-labeling agents that effectively inhibit the CoA transport system provides important tools which will facilitate the measurement of CoA transport in both isolated mitochondria as well as in reconstituted proteoliposomal systems. Along these lines, experiments are currently in progress which seek to extract the CoA transporter from the mitochondrial inner membrane and reconstitute its function in phospholipid vesicles.

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