Enzymatic formation of glutathione-citryl thioester by a mitochondrial system and its inhibition by (-)erythrofluorocitrate

(glutathione-S-citryl ester/metalloprotein/inner mitochondrial membrane/fluorocitrate toxic mechanism)

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ABSTRACT A soluble extract of the mitochondrial compartment composed of the inner membrane and matrix catalyzes the enzymatic synthesis and hydrolysis of the 1:1 adduct of citric acid and glutathione. The adduct was identified as the thioester by isolation with single and double isotope labeling (14C)citric acid and (32P)glutathione and by conversion to the monohydrate of citric acid and synthesis of the synthetic product by thin layer chromatography and high voltage electrophoresis. The enzymatic formation of the thioester (pH optimum 7.39 at 30°) requires oxidized glutathione and citrate; both substrates exhibit a Michaelis– Menten kinetics. During the enzymatic reaction equimolar quantities of thioester and glutathione sulfenic acid are formed. After gel filtration or salt fractionation the enzyme system requires Mn2+ (or Mg2+, which is less effective) for maximal activity. When extracts of mitoplast are tested, the time course of reaction is biphasic due to the rapid synthesis of the product by the thioester-forming system (molecular weight 171,000) followed by its decay by the hydrolyase (molecular weight 71,000). The two systems were separated by molecular filtration on Sephadex G-500 and by precipitation with (NH4)2SO4. The thioester-forming system is inhibited by preincubation with 0.5 mM mersalyl. Other inhibitors are 1,2,2-propane tricarboxylic acid, 10 mM Ca2+, 200 mM K+, and the free radical trapping agent, phenazine methosulfate. The citrate-glutathione thioester formation is irreversibly and specifically inhibited by (-)erythrofluorocitrate (50% inhibition at 25 pmol of added fluorocitrate per mg of protein), which forms a trichloroacetic acid-stable adduct with the enzyme protein (at 5% inhibition, 0.8 pmol is bound to 1 mg of protein). Synthesis of malyl-glutathione thioester by inner membrane vesicles is selectively inhibited by (-)erythrofluoromalate.

We have previously identified the biologically active species of the four possible isomers of monofluorocitric acid as (-)erythrofluorocitrate (1, 2); its structure subsequently was confirmed by x-ray crystallography (3, 4). Kinetic evidence indicated that (-)erythrofluorocitrate, when preincubated with isolated mitochondria at concentrations of 20–50 pmol of fluorocitrate per mg of mitochondrial protein, irreversibly inhibited bidirectional citrate transport (5–8). Whereas the high sensitivity of citrate influx to low concentrations of fluorocitrate in intact mitochondria has been confirmed (9), an unusually low competitive k1 value for fluorocitrate was also reported for the aconitase activity of disrupted mitochondria that had been preincubated with 1 mM Mg2+ (9). However, we have shown subsequently that mitochondrial aconitase is inactivated by Mg2+ in the presence of low concentrations of fluorocitrate (10) and apparent k1 values calculated under these conditions have no bearing on the linearly competitive and reversible inhibition of purified aconitase by (-)erythrofluorocitrate (k1 = 290 μM; see ref. 11). The inability to demonstrate an inhibition of citrate efflux by fluorocitrate in mitochondria that had been loaded with citrate (9) was also explained by the stringent requirement of preincubation of mitochondria with fluorocitrate in the absence of millimolar concentrations (cf. 7, 8), allowing for the formation of two protein–fluorocitrate thioesters (8).

The present report is concerned with the identification of a mitochondrial enzyme system that catalyzes the formation of the 1:1 adduct of glutathione and citric acid. The adduct was identified as the thioester of glutathione, and kinetic evidence was also obtained that the mitochondrial extract contained, in addition to the thioester-forming enzyme system, a hydrolase capable of degrading the thioester. The citryl-glutathione-forming enzyme system proved to be the most sensitive catalytic component of mitochondria towards the irreversible inhibitory action of (-)erythrofluorocitrate, suggesting that the molecular site of action of this highly toxic agent has been identified.

METHODS

Lysosome-free mitochondria and mitoplasts (defined as the matrix surrounded by the inner membrane) were isolated from rat livers as described (12). An extract of mitoplasts was prepared by stirring (at 0°) of a suspension of mitoplasts (30 mg of protein per ml) in 0.25 M sucrose/mannitol medium (12) containing 1 mg of Brij 56 (Sigma Chemicals) per 10 mg of mitoplast protein for 15 min. The resulting lysate was diluted 1:3 with the medium; after removal of large granules (unbroken mitoplasts) by centrifugation at 10,000 X g (10 min at 4°), the supernatant (containing soluble extract of the inner membrane and soluble proteins of the matrix and inner membrane vesicles) was subjected to ultracentrifugation (145,000 X g for 1 hr at 2°). The clear supernatant was concentrated by centrifugation (at 2°) in Amicon CF-25 membrane cones at 100 X g to one-fourth of the original volume. Approximately 250 mg of protein extract was obtained by this technique from 500 mg (protein) of mitoplast starting material. The concentrated extract was used directly in most experiments, unless specified, whereas the sediment containing the inner membrane vesicles was rehomogenized in the suspending medium. Both fractions were stored at −15° until used. The enzymatic activity of the soluble extract decayed slowly at −15° (about half of the activity was lost in 2 weeks) but much more rapidly after repeated thawing and refreezing. The activity was completely recovered when the stored extract was preincubated for 5–10 min with 5 mM reduced glutathione (GSH), demonstrating the essential role

Abbreviations: GSH, reduced glutathione; GSSG, oxidized glutathione; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; citryl-SC and malyl-SC, glutathione thioesters of citric and malic acids, respectively.
of protein-bound -SH groups for catalytic activity. As shown later, GSH was not a substrate of the enzyme, but served as a thiol-protecting agent.

The glutathione-citrate adduct was quantitatively assayed by its isolation as follows. After incubation of the enzyme with oxidized glutathione (GSSG), GSH, and citrate (for details see Results) containing [1,5-14C]citric acid (98 mCi/mmol; New England Nuclear) in a final volume of 100 μl, the reaction was stopped with 300 μl of 10% trichloroacetic acid and the precipitated proteins were removed by centrifugation. An aliquot of the trichloroacetic acid supernatant (300 μl) was directly transferred onto a small column (1 × 2 cm, containing 1.5 ml of resin bed) of Dowex 50 H+ equilibrated with 1 M formic acid/Na formate of pH 2.0 and the unreacted citric acid was quantitatively removed by 12 ml of formic acid (1 M, pH 2.0), as monitored by radiochemical analysis. After the residual formic acid was eluted from the column with 3 ml of H2O, the adduct was quantitatively eluted with 6 ml of 1 M NaOH and an aliquot (1.5 ml), after neutralization with 1 M HCl, was analyzed by scintillation spectrometry with toluene/Triton X-100/Omnifluor (1 liter:1 liter:18 g) scintillator. Labeled GSH (35S, 19 mCi/mmol, obtained from Schwartz/Mann) was used as the second label for the product. Hydroxamate derivatives were prepared from synthetic monomethyl ester of citric acid (prepared by the diazomethane procedure, with 0.9 mol of diazomethane per mol of citrate in methanol) and from the enzymatically synthesized product by reacting it directly (after freeze-drying) with a methanolic solution of NH2OH (13) for 1 hr at 40° after 16 hr at room temperature. Hydroxylaminolysis was 60% under these conditions. The excess reagent was removed by freeze-drying. Thin layer chromatography was carried out on silica gel sheets (Eastman) with butanol/acetic acid/H2O (4:1:1) as developing solvent. The technique of high voltage electrophoresis on Whatman no. 3 paper and analytical methods have been described (14). (=)-Erythrofluorono-[3-614C]citric acid was synthesized by enzymatic condensation of [1-414C]oxaloacetic acid with synthetic fluorooxoacetyl-coenzyme A (1, 2). The products was purified by high voltage electrophoresis at pH 1.85, resulting in homogeneous fluorocitrate (50 mCi/mmol). Unlabeled (=)-erythrofluoromalate was synthesized as described (15).

RESULTS

The electrophoretic isolation of the enzymatically formed citrate-glutathione adduct is shown in Fig. 1. A typical reaction mixture contained 1.76 mg (protein) of mitoplast extract, 5 mM GSH, 15 mM GSSG, 220 μM citrate, Tris/N-2-hydroxethylpiperazine-N'-2-ethanesulfonic acid (Hepes) buffer (10 mM, pH 7.4), and sucrose mannitol (0.25 M) in a final volume of 600 μl. After incubation for 5 min at 30°, the reaction was stopped with 2 ml of 10% trichloroacetic acid. The product was adsorbed to Dowex 50 H+ (see Methods) from six 100-μl batches of the incubation mixture and eluted five times with 1 M pyridine (2 ml for each batch). The pyridine eluates were collected into one-half volume of 2 M acetic acid (at 0°) in order to avoid alkali-catalyzed rearrangement of the thioester (16) and freeze-dried, and the product was isolated by high voltage electrophoresis at pH 3.5 (0.2 M acetate/formate) and 4000 V for 150 min at 20° (14). In the experiment shown in Fig. 1 either citrate, 14C, or glutathione, 35S, was labeled. Both labels appeared in the same radioactive peak, corresponding to a 1:1 molar ratio of citric acid and GSH. From the above large-scale preparative experiment 200 nmol of citrate glutathione (1:1) adduct was obtained, which was used in part for conversion to the hydroxamate (see Methods). Comparison of the NH2OH derivative of the enzymatically synthesized product and the hydroxamate prepared from the chemically synthesized monomethyl ester of citric acid showed that the two products migrated with identical mobility, Rp of 0.27, whereas Rp of the unreacted citric acid was 0.18 and the Rp of the 1:1 adduct was 0.36. The hydroxamic acid derivatives of citric acid, obtained either from the monomethyl ester or from the enzymatic product, were also electrophoretically separated at pH 1.85 (1.0 M formic acid). Both hydroxamic acid derivatives moved towards the cathode at a rate of 2 cm/hr, whereas citric acid remained at the origin. Both synthetic and enzymatically formed hydroxylamine derivatives gave a reddish-violet spot on the silica plate when sprayed with ethanolic FeCl3, typical of hydroxamic acids. These results identified the enzymatic product as citryl-glutathione thioester.

The time course of the formation of the GS-citric acid thioester catalyzed by the unfraccionated extract of mitoplasts was characteristically biphasic (Fig. 2), indicating a rapid synthesis and, at a critical concentration of the product, a progressive hydrolysis to GSH and citric acid. It is also apparent that the enzyme system is predominantly in the extract and only traces of the thioester synthetase remain in membrane vesicles. Protein fractions containing either synthetic or hydrolytic activities were readily separated from the extract of mitoplasts by molecular filtration on Sephadex G-200, developed with 50 mM Tris/Hepes, pH 7.4 at 4°. The citryl-SG synthetase activity appeared in the molecular fraction exhibiting a mass of 171,000 daltons, whereas the hydrolase had a molecular mass of 71,000.
daltons. These protein fractions represent a 450- and 250-fold purification, respectively, as compared to total liver tissue. Recombination of the two protein fractions reconstituted the biphasic time course characteristic for the unfractionated extract. The time course of the reaction catalyzed by the protein fraction of 171,000 daltons was sustained for hours provided 0.5 mM MnCl₂ was also present (Fig. 3). These results indicate that molecular filtration rendered the citryl-glutathione-synthesizing enzyme system dependent on Mn²⁺. Separation of the thioester synthetase system from the hydrolyase was also accomplished by fractionation with (NH₄)₂SO₄. The synthetase system was recovered in the fraction between 0 and 35% (wt/vol). This preparation also exhibited dependence on Mn²⁺ for the synthesis of the thioester. No other cation except Mg²⁺ could replace Mn²⁺; Mg²⁺ was somewhat less effective, and Ca²⁺ was inhibitory. The enzyme system was inhibited 80% by 200 mM K⁺. Since intramitochondrial concentration of K⁺ in the matrix can be as high as 180 mM (12), it is probable that variation in the mitochondrial K⁺ content exerts a regulatory effect on the activity of this enzyme system. A typical inhibitor of mitochondrial citrate translocation, 1,2,3-propane tricarboxylate (17–19), at 1 mM concentration inhibited the for-}

**Fig. 2.** The time course of the enzymatic formation of GS-citryl thioester catalyzed by an extract of mitoplast and by inner membrane vesicles. Amount of protein per test, 0.7 mg; GSH, 10 mM; GSSG, 10 mM; citrate, 100 μM; 30°C, pH 7.4. Total volume, 100 μl. Small nonenzymatic rates were always subtracted. GS-citrate, citryl glutathione thioester.

**Fig. 3.** The effect of 0.5 mM MnCl₂ on the rates of GS-citryl thioester formation by a protein fraction (molecular weight 171,000) of the extract of mitoplasts obtained by molecular filtration on Sephadex G-200; 0.35 mg of protein per test system. Conditions were the same as described in the legend of Fig. 2.

**Table 1. Effects of activators and inhibitors on enzymatic formation of GS-citryl thioester**

<table>
<thead>
<tr>
<th>Inhibitors or activators</th>
<th>pmol GS-thioester formed/mg protein in 5 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>2180</td>
</tr>
<tr>
<td>0.5 mM Mn²⁺</td>
<td>4260</td>
</tr>
<tr>
<td>1 mM Mg²⁺</td>
<td>3770</td>
</tr>
<tr>
<td>1 mM Ca²⁺</td>
<td>1660</td>
</tr>
<tr>
<td>10 mM Ca²⁺</td>
<td>80</td>
</tr>
<tr>
<td>1 mM 1,2,3-Propane tricarboxylate</td>
<td>405</td>
</tr>
<tr>
<td>1 mM Phenazine methosulfate</td>
<td>362</td>
</tr>
<tr>
<td>200 mM KCl</td>
<td>471</td>
</tr>
<tr>
<td>0.5 mM Mersalyl (preincubation)</td>
<td>104</td>
</tr>
</tbody>
</table>

An extract of mitoplast (0.4 mg of protein) was incubated with 10 mM GSH, 10 mM GSSG, and 14C-labeled citrate (final molarity of 100 μM) for 5 min in the presence of the agents listed. The effect of mersalyl (0.5 mM) was tested by preincubation for 5 min prior to the enzymatic assay.

The enzymatic synthesis of citryl-glutathione thioester would be expected to be an energy-requiring process; therefore a simultaneous oxidation of S in glutathione to a higher oxidation state was anticipated. Sulfinic acid of cysteine is not adsorbed on Dowex 50 H⁺ under our conditions (20, 21); therefore this technique provided a quantitative assessment of the formation of glutathione sulfenic acid during the synthesis of citryl-glutathione thioester. Within an experimental error of ±12%, 1 mol of sulfenic acid was generated for each mole of thioester formed, indicating the simultaneous contribution of an oxygenase type of reaction (21) coincident with the formation of the citryl-glutathione thioester. During all enzymatic reactions reagent blanks showed the formation of a small but measurable quantity of thioester (0.5–1% of the enzymatic rate) and significant quantities of sulfinic acid. Sulfinic acid was detectable in an aqueous solution of GSSG (about 8%). The rates in the reagent blanks exhibited no temperature dependence between 0° and 40°, while the enzymatic rates were more than doubled for each 10° within this temperature range. The pH optimum of the synthetase reaction was 7.39 at 30°.

Typical Michaelis–Menten kinetics was obtained with citrate (Fig. 4) and GSSG (Fig. 5). The concentration of GSSG was maintained at 10 mM for the determination of the apparent K₉ₐ of citrate because this concentration of glutathione corresponded to known intracellular concentrations of total glutathione (22). Since the apparent K₉ₐ for GSSG was 9 mM (at a fixed concentration of citrate of 220 μM), it is evident that the rate of reaction is very sensitive to fluctuations in GSSG concentrations, suggesting that the intracellular GSSG/GSH ratio and the absolute concentration of glutathione are powerful cellular regulators of this enzyme system.

When the extract of mitoplasts was preincubated with increasing concentrations of 14C-labeled (--)erythrofluorocitrate for 5 min at 30°, the rates of formation of the GS-citrate thioester were progressively inhibited (Fig. 6). The protein-
bound fluorocitrate was simultaneously determined by precipitation of the protein with 10% trichloroacetic acid and radiochemical assay for bound fluorocitric acid by the glass fiber filtration technique (14). A correlation was found between the degree of inactivation of the GS-citryl thioester synthase system and the quantity of acid-stable protein-fluorocitrate product. About 50% inactivation occurred when 0.8 pmol of fluorocitrate were bound to 1 mg of protein, corresponding to 25 pmol of added fluorocitrate per mg of protein. The formation of the trichloroacetic acid-stable protein adduct of fluorocitrate continued at a linear rate even after almost complete inhibition of the synthetase. This phenomenon is readily explained by the fact that fluorocitrate was bound not only to the synthetase, but also to the hydroxase, the latter being present in 3-fold excess over the synthetase in the extract. The maximal capacity of the extract to bind fluorocitrate in a trichloroacetic acid-stable form was 80 pmol/mg of protein, corresponding to the total amount of reactive sites in both enzymes. Synthetic (-)erythrofluoromalate (15), even at millimolar concentrations, had no effect on the citryl-SG thioester-synthesizing system. On the other hand, the formation of malyl-glutathione thioester, a reaction catalyzed by inner membrane vesicles, was selectively inhibited by fluoromalate but not by fluorocitrate. These results are shown in Table 2. The formation of malyl-SG or of other carboxylic acid thioesters was assayed by the same method as described for citryl-glutathione except that L-[14C]malate or other carboxylic acids were substituted for citrate. It is important that the malyl-SG-synthesizing system remained attached to the inner membrane vesicles (see Table 2), whereas the citryl-SG synthetase system was extracted by the technique used (see Methods; compare Table 2 with Fig. 2). Experiments completed at the present time show that specific enzyme systems exist in mitochondria that catalyze the GS-thioester formation of succinic, malic, α-ketoglutaric, glutamic, pyruvate, and isocitric acids and of glutamine; thus, the reaction described for citrate may have general metabolic significance.

**DISCUSSION**

The two mitochondrial proteins that form thioesters with (-)erythrofluoromalate (8) were identified as citryl-S-gluta-

**Table 2. Effects of fluorocitrate and fluoromalate on GS thioester-synthetase activities of submitochondrial preparations with citrate and L-malate as substrates.**

| Inhibitors | 
|---|---|---|
| Fluorocitrate | Fluoromalate | 
| 125 pmol/| 50 nmol/ | 
| mg protein | mg protein | 
| Extract + citrate | 2770 | 110 | 2802 |
| Membrane vesicles + L-malate | 305 | 298 | 0 |

Conditions for the assay for GS-citryl thioester formation were the same as described in the legend of Fig. 2, except that the extract (1 mg of protein) was preincubated with or without the inhibitors for 10 min at 30°. The concentration of L-malate (77,000 cpm/nmol) was 68 μM in the GS-malyl synthetase system; otherwise, conditions were the same as for the GS-citryl thioester-forming system.

* Results are expressed as pmol of thioester formed in 5 min per mg of protein.
thione synthetase and hydrolase, an observation that suggests that new sites of action of this toxic agent have been identified. The concentrations of fluorocitrate that inhibit citrate transport and the citrly-S-glutathione enzymes are of the order of magnitude found in mitochondria of lethally poisoned rats (23). The exact role of the citrly-S-glutathione enzymes in mitochondrial metabolism is unknown, but at least two possible functions may be postulated. The thioester synthetase is inhibited by 1,2,3-propane tricarboxylate (Table 1), which is generally recognized as a specific inhibitor of citrate transport in intact mitochondria (17-19). Coincidence of inhibition of citrate transport in mitochondria with inhibition of the soluble citrly-S-glutathione thioester synthetase tends to suggest that the thioester synthetase may be a constituent of the mitochondrial transport system. It has been reported that various dicarboxylic acids exhibit competitive inhibition with respect to citrate uptake in intact mitochondria (18). This apparent competition can be readily demonstrated with inner membrane vesicles containing all di- and tricarboxylic acid-S-glutathione thioester synthetases, when a limiting concentration of GSSG is exposed simultaneously to citric acid and dicarboxylic acids. In this system apparent competition for GSSG as the second substrate of the reaction takes place. The citrate-malate exchange diffusion in mitochondria has been reported to be sensitive to thiol reagents (24), but observations to the contrary also exist (25), whereas the soluble thioester synthetase requires preincubation with mersalyl for inhibition (Table 1). Protein-thiol groups, which are essential for citrate transport, may not be readily accessible to thiol reagents; this inaccessibility can explain variable results. It has been shown that glutamate-aspartate exchange is not sensitive to theic thiol reagents, but is inhibited by uncharged -SH reagents (26). The apparent $V_{max}$ for citrate-malate exchange was calculated to be 7-5* nmol/mg of protein per min (18). The $V_{max}$ estimated from Fig. 5 for the soluble synthetase can account for only 15 of citrate transport in intact mitochondria. However, the $V_{max}$ of the soluble enzyme was found to be increased by 3-fold (on a mg of protein basis) when membrane vesicles were added back to the soluble enzyme, demonstrating that nonenzymatic membrane constituents, e.g., lipids, have a decisive effect on the rate of enzymatic catalysis. Therefore, the efficiency of the soluble enzyme for citrate transport cannot be estimated at this time. The apparent $K_m$ for citrate in intact mitochondria was calculated from 20- to 60-sec rates at 9" to be 120 μM (15), whereas the apparent $K_m$ of the soluble enzyme determined at 10 mM GSSG from 5-min rates at 30° was 56 μM (Fig. 4). Differences in these values are probably due to different experimental conditions.

A second possible mitochondrial role of the glutathione carboxylic acid thioester enzyme system can be predicted from present results and may explain a special function of the unusually high cellular concentration of glutathione (22). The concentration of all free carboxylic acid substrates of the Krebs cycle can be directly regulated by the thioester synthetase-hydrolase system, an effect that may profoundly influence cellular metabolism. The existence of glutathione S-formyl and succinyl thioesters in human liver has been demonstrated, but no biochemical role for these substances has been proposed (27, 28). The enzymatic formation of thioesters involves GS' formed by the homolytic cleavage of GSSG. The existence of GS' is well documented (29).

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