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THE TRANSPORT OF L-CYSTEINESULFINATE IN RAT LIVER MITOCHONDRIA

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Summary

1. The mechanism of L-cysteinesulfinate permeation into rat liver mitochondria has been investigated.

2. Mitochondria do not swell in ammonium or potassium salts of L-cysteinesulfinate in all the conditions tested, including the presence of valinomycin and/or carbonylcyanide *p*-trifluoromethoxyphenylhydrazone.

3. The activation of malate oxidation by L-cysteinesulfinate is abolished by aminooxyacetate, an inhibitor of the intramitochondrial aspartate aminotransferase, it is not inhibited by high concentrations of carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (in contrast to the oxidation of malate plus glutamate) and it is decreased on lowering the pH of the medium.

4. All the aspartate formed during the oxidation of malate plus L-cysteinesulfinate is exported into the extramitochondrial space.

5. Homocysteinesulfinate, cysteate and homocysteate, which are all good substrates of the mitochondrial aspartate aminotransferase, are unable to activate the oxidation of malate. Homocysteinesulfinate and homocysteate have no inhibitory effect on the L-cysteinesulfinate-induced respiration, whereas cysteate inhibits it competitively with respect to L-cysteinesulfinate.

6. In contrast to D-aspartate, D-cysteinesulfinate and D-glutamate, L-aspartate inhibits the oxidation of malate plus L-cysteinesulfinate in a competitive way with respect to L-cysteinesulfinate. Vice versa, L-cysteinesulfinate inhibits the influx of L-aspartate.

7. Externally added L-cysteinesulfinate elicits efflux of intramitochondrial

Abbreviations: MalNEt, *N*-ethylmaleimide; Mops, 3-(*N*-morpholino)propanesulfonic acid; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; TMPD, *N,N,N′,N′*-tetramethyl-1,4-phenylenediamine.

L-aspartate or L-glutamate. The cysteinesulfinate analogues homocysteinesulfinate, cysteate and homocysteate and the D-stereoisomers of cysteinesulfinate, aspartate and glutamate do not cause a significant release of internal glutamate or aspartate, indicating a high degree of specificity of the exchange reactions. External L-cysteinesulfinate does not cause efflux of intramitochondrial P_i, malate, malonate, citrate, oxoglutarate, pyruvate or ADP. The L-cysteinesulfinate-aspartate and L-cysteinesulfinate-glutamate exchanges are inhibited by glisoxepide and by known substrates of the glutamate-aspartate carrier.

8. The exchange between external L-cysteinesulfinate and intramitochondrial glutamate is accompanied by translocation of protons across the mitochondrial membrane in the same direction as glutamate. The L-cysteinesulfinate-aspartate exchange, on the other hand, is not accompanied by H^+ translocation.

9. The ratios $\Delta H^+/\Delta$glutamate, ΔL-cysteinesulfinate/Δglutamate and ΔL-cysteinesulfinate/Δaspartate are close to unity.

10. It is concluded that L-cysteinesulfinate is transported by the glutamate-aspartate carrier of rat liver mitochondria. The present data suggest that the dissociated form of L-cysteinesulfinate exchanges with H^+-compensated glutamate or with negatively charged aspartate.

Introduction

L-Cysteinesulfinate is an intermediate produced during the degradation of cysteine to sulfate [1]. In 1956 it was shown [2] that in the presence of mitochondrial acetone powders L-cysteinesulfinate transaminates with α-oxoglutarate or oxaloacetate to yield glutamate or aspartate, and β-sulfinylpyruvate, which spontaneously hydrolyses into sulfite and pyruvate. Since this observation L-cysteinesulfinate has been extensively used in studies with mitochondria from different tissues [3—14]. Although L-cysteinesulfinate is actively metabolized by intact mitochondria the question as to how it enters the mitochondria has not yet been considered.

The present paper examines the permeation of L-cysteinesulfinate in rat liver mitochondria and presents evidence that this amino acid may be transported by the glutamate-aspartate carrier in exchange for either aspartate or glutamate. The L-cysteinesulfinate-glutamate exchange is electrogenic since the efflux of glutamate is H^+ compensated, whereas the L-cysteinesulfinate-aspartate exchange is electroneutral. A preliminary account of part of this work has been communicated [15].

Materials and Methods

Materials. L-[G-^{3}H]Aspartic acid, L-[G-^{3}H]glutamic acid, [^{32}P]phosphoric acid, [U-^{14}C]sucrose, ^3H_2O, [1-^{14}C]malonate, [U-^{14}C]malate, [1,5-^{14}C]citrate, [5-^{14}C]oxoglutarate, [U-^{14}C]pyruvate and [2-^{3}H]ADP were obtained from the Radiochemical Centre (Amersham, U.K.), antimycin A, MalNEt, mersalyl (*o*-(3-hydroxymercuri-2-metoxypropyl)carbamoylphenoxyacetate) and bathophenanthroline from Sigma, benzene-1,2,3-tricarboxylate from Schuchardt,

L-*allo*-γ-hydroxyglutamic acid, L-α-aminoadipic acid, α-aminooxyacetic acid and valinomycin from Calbiochem, D-aspartic acid, D-glutamic acid, *meso*-diaminosuccinic acid and α-aminopimelic acid from Fluka, *n*-butylmalonic acid from Aldrich, dextran T 70 (mol. wt. about 70 000) from Pharmacia. Rotenone was kindly supplied by F.P. Penick and Co. (New York), glisoxepide by Bayer, DL-cycloserine by Dr. V.P. Skulachev, FCCP by Dr. P. Heytler (du Pont), nigericin by Dr. H.A. Lardy, α-cyanocynnamate by Dr. G. Paradies, L-cysteic acid, taurine and ipotaurine by Dr. R. Scandurra, D-cysteinesulfinic acid and DL-homocysteic acid by Dr. C. De Marco, *N*-benzoylaspartic acid by Dr. G. Marcotrigiano and phthalonate by Dr. L. Troisi. L-Cysteinesulfinic acid was synthetized from L-cystine according to the method of Lavine [16]. L-[^{3}H]-Cysteinesulfinate was prepared from L-[3,3'-^{3}H]cystine, obtained from the Radiochemical Centre. L-Homocysteinesulfinic acid was synthetized according to the method of Luchi and De Marco [17].

Rat liver mitochondria were isolated as previously reported [18].

Loading of mitochondria. The procedure for loading the mitochondria with glutamate was essentially similar to that described [19,20] with some modifications advised by K.F. LaNoue (personal communication). Mitochondria (30—50 mg protein) were incubated at 0° C in 5 ml medium consisting of 100 mM glutamate, 100 mM sucrose, 5 mM NH_4Cl, 5 mM ascorbate, 200 μM TMPD, 0.5 μg/mg protein rotenone and 10 mM Mops/KOH at pH 6.8. After 5 min, 100 nmol/mg protein MalNEt was added to inhibit the glutamate-OH carrier [19,21]. Then, the mitochondria were washed twice in 140 mM sucrose, 140 mM mannitol, 2 μg/ml rotenone and 10 mM Mops at pH 7.0, suspended at 70—80 mg protein/ml in the same medium and used as quickly as possible, generally within 1 h. After this loading procedure, the intramitochondrial content of glutamate varied between 6 and 11 nmol/mg protein. In some experiments, the intramitochondrial glutamate was labelled by adding to the mitochondrial suspension carrier-free [^{3}H]glutamate (approximately 2 μCi/ml of mitochondrial suspension). Equilibration of the radioisotope between the extramitochondrial and the intramitochondrial pools of glutamate was obtained after 2 min incubation at 10° C.

Mitochondria were loaded with aspartate by the method introduced by LaNoue et al. [20] according to the following procedure. Glutamate-loaded mitochondria (70—80 mg protein) were incubated at 20° C in 10 ml of a medium consisting of 140 mM sucrose, 140 mM mannitol, 2 μg/ml rotenone and 10 mM Mops, pH 7.0, to which 3 mM oxaloacetate and 1 mM MalNEt were added. After 2 min, 2 mM aminooxyacetate was added to inhibit the aspartate aminotransferase [22,23]. After one further min, the volume was made up to 50 ml with the above ice-cold medium, and the mitochondria were separated by centrifugation and suspended in the same medium with addition of 2 mM aminooxyacetate, at a concentration of 40—50 mg protein/ml. In some experiments, the mitochondria were loaded with labelled aspartate by incubating aspartate-loaded mitochondria for 2 min at 10° C with carrier-free [^{3}H]aspartate (approximately 2 μCi/ml of mitochondrial suspension). The intramitochondrial content of aspartate varied between 4 and 8 nmol/mg protein.

The procedures for loading the mitochondria with labelled P_i, malate,

malonate, citrate, oxoglutarate, pyruvate and ADP were previously described [24].

Measurement of anion distribution between the inner and the outer mitochondrial phase. Mitochondria were incubated under the conditions specified in the legends and separated from the incubation mixture by centrifugal filtration through a layer of silicone [24]. The metabolites were assayed in the extracts of the sediments and supernatants enzymatically or radioactively, and their content in the intramitochondrial space was calculated as previously described [25]. [^{14}C]Sucrose was used to correct for the anion present in the unspecifically permeated space.

Measurement of the exchange between intra- and extramitochondrial metabolites. The efflux of labelled metabolites from previously loaded mitochondria was initiated by the addition of appropriate counteranions to the incubation medium (back exchange). Control experiments, i.e. in the absence of external anion, were performed in order to correct for the small time-dependent spontaneous leakage of the metabolite. At the end of the incubation the mitochondria were separated by centrifugation for 1 min in an Eppendorf bench centrifuge (model 3200). The percentage exchange was calculated by one of the following two methods: (a) from the radioactivity in the pellet according to the equation: percent exchange = 100 ($cpm_{control} - cpm_{assay}$)/$cpm_{control}$, where cpm represents the radioactivity in the pellet extracts; (b) from the radioactivity released in the supernatant according to the equation:

$$\text{Percent exchange} = 100 \cdot \frac{cpm_{assay} - cpm_{control}}{cpm_{total} - cpm_{control}}$$

where the 'assay' and 'control' values are obtained from supernatants, and the 'total' from uncentrifuged samples. In some cases, the metabolites were assayed enzymatically.

Other methods. The rate of mitochondrial swelling was monitored by recording the decrease in the absorbance at 546 nm.

The oxygen uptake was measured polarographically with a Clark electrode, using 2—4 mg mitochondrial protein in a final volume of 1.3 ml at 25° C. Rates of oxygen consumption are expressed in ng atoms/min per mg protein.

Changes in the redox state of intramitochondrial nicotinamide nucleotides were followed by fluorimetry using an Eppendorf 1101 M fluorimeter. Filters of 313 plus 366 nm and 470—3000 nm were used for excitation and emission light, respectively.

The pH of the medium was monitored potentiometrically using a fast responding, low resistance glass electrode (Ingold combination electrode type 104-02-3010) connected to an expanded scale pH meter (Radiometer, Copenhagen, Model 27), the output of which drove a recorder (Honeywell). The potential changes were quantitated in terms of proton equivalents by double titration with standard solutions of KOH and HCl. The mitochondria used for this type of experiment were washed and resuspended in a medium containing 1 mM Mops instead of 10 mM.

The pK values of L-cysteinesulfinic acid and L-homocysteinesulfinic acid were determined by titration with 1 M NaOH in N_2-saturated media (0.2 M solutions at 20° C). The following values were found: $pK_1 = 1.50$, $pK_2 = 2.38$

and $pK_3 = 9.24$ for cysteinesulfinic acid, and $pK_1 = 1.66$, $pK_2 = 2.60$ and $pK_3 = 9.35$ for homocysteinesulfinic acid.

The mitochondrial protein was determined by the biuret method after being dissolved with sodium cholate, using bovine serum albumin as standard.

Glutamate [26], aspartate [26] and pyruvate [27] were assayed enzymatically, using the double-beam spectrophotometer and a wavelength pair of 350 and 375 nm, or a Cary spectrophotometer model 118 CX at 340 nm. The assays of aspartate in media containing aminooxyacetate were carried out in the presence of 5 mM acetaldehyde [20]. Sulfite was determined colorimetrically at 584 nm according to the method of Grant [28].

The radioactivity was measured in a Packard liquid scintillation counter in the 1,4-bis-(5-phenyloxazolyl-2)-benzene and 2,5-diphenyloxazole system.

Results

Mitochondrial swelling

In experiments not shown, we have found that rat liver mitochondria do not swell in ammonium L-cysteinesulfinate under the conditions in which they swell in ammonium P_i or ammonium acetate [29]. Nor does mitochondrial swelling occur in ammonium L-cysteinesulfinate, after the addition of catalytic concentrations of P_i or P_i plus malate, which are required for swelling in ammonium malate or ammonium citrate, respectively [29]. We have also found that mitochondria, made permeable to K^+ by valinomycin or to both K^+ and H^+ by nigericin or by the association of valinomycin and FCCP, do not swell in potassium L-cysteinesulfinate. These results indicate that L-cysteinesulfinate is not transported via a uniport, either electroneutrally (together with H^+) or electrogenically. They strongly suggest that L-cysteinesulfinate, if permeable across the mitochondrial membrane, is transported by an exchange mechanism involving the movement of another metabolite in the opposite direction.

Oxidation of malate plus L-cysteinesulfinate

Fig. 1 (Expts. 1—3) shows the effect of transaminase inhibitors on the oxidation of malate plus L-cysteinesulfinate. The addition of DL-cycloserine, an inhibitor of the aspartate aminotransferase which cannot penetrate the mitochondrial membrane [23,30], does not affect the L-cysteinesulfinate-induced respiration (Expt. 2). On the other hand, aminooxyacetate, which inhibits both the cytosolic and the mitochondrial aspartate aminotransferase [22,23], completely abolishes the stimulation of respiration caused by L-cysteinesulfinate (Expt. 3). These results indicate that L-cysteinesulfinate enters the mitochondria and reacts with the intramitochondrial transaminase, removing oxaloacetate and hence stimulating malate oxidation.

Since it is known that the oxidation of glutamate, which also involves a transamination with oxaloacetate, is inhibited by high concentrations of uncouplers [9,31—33], the effect of increasing concentrations of FCCP on the oxidation of malate plus L-cysteinesulfinate was investigated. As illustrated in Fig. 2A, the uncoupler, even at very high concentrations, has no inhibitory effect on the respiration supported by malate plus L-cysteinesulfinate, whereas it has a marked effect on the oxidation of malate plus glutamate. Fig. 2B illus-

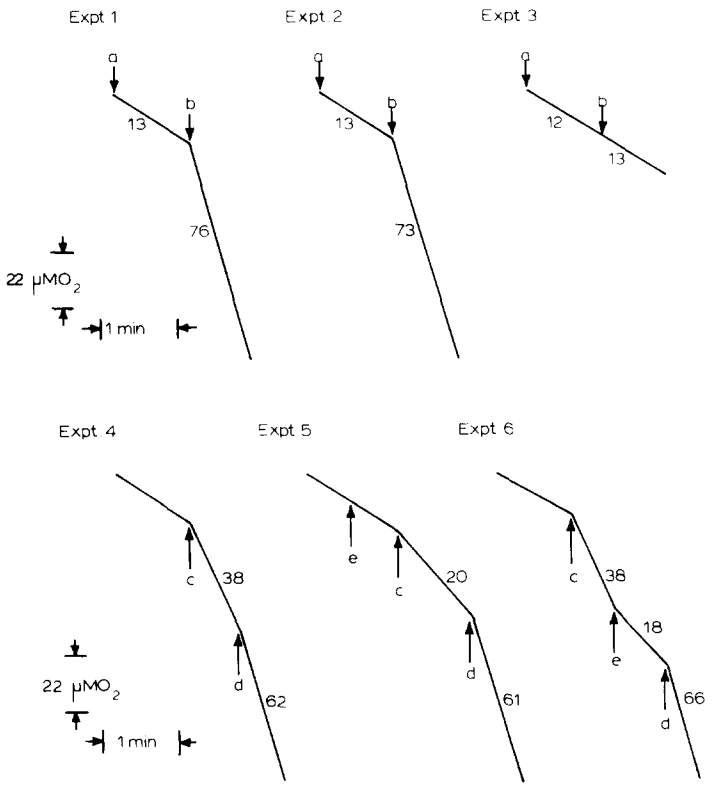

Fig. 1. Effect of DL-cycloserine, aminooxyacetate and L-aspartate on mitochondrial respiration supported by malate plus L-cysteinesulfinate. Expts. 1—3: the reaction mixture contained 125 mM KCl, 10 mM Tris-HCl, pH 7.4, 5 mM P_i, 2 mM ADP, 3.2 mg mitochondrial protein, and in Expt. 2 5 mM DL-cycloserine, in Expt. 3 3 mM aminooxyacetate. Additions: 5 mM malate at points a and 7.5 mM L-cysteinesulfinate at points b. Expts. 4—6: the incubation mixture contained 300 mM sucrose, 10 mM Tris-HCl, pH 7.4, 5 mM P_i, 1 μM FCCP, 7.5 mM malate and 3.3 mg mitochondrial protein. Additions: 0.75 mM L-cysteinesulfinate at points c, 10 mM L-cysteinesulfinate at points d, and 2.5 mM L-aspartate at points e.

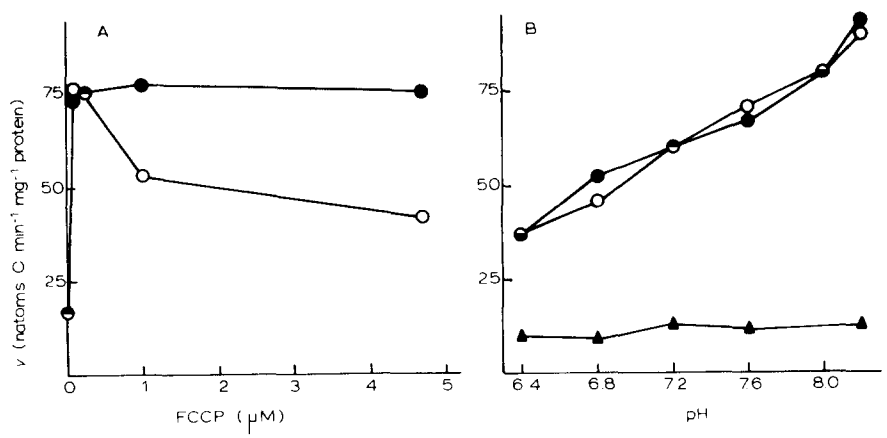

Fig. 2. Effect of increasing concentrations of FCCP (A) and of external pH (B) on the oxidation of malate plus L-cysteinesulfinate. The incubation mixture contained 125 mM KCl, 10 mM Tris-HCl, 5 mM P_i, FCCP at the concentrations indicated, 7.5 mM malate, 3.6 mg protein and 10 mM L-cysteinesulfinate (●), or 10 mM L-glutamate (○) in (A), and 300 mM sucrose, 10 mM Mops/Tris, 5 mM P_i, 0.4 μM FCCP, 5 mM malate, 7.5 mM L-cysteinesulfinate and 3.8 mg protein in (B). The final pH was 7.4 in (A) and as indicated in (B). In (B): ●, complete system; ○, without P_i; ▲, without L-cysteinesulfinate.

TABLE I

DISTRIBUTION OF ASPARTATE PRODUCED DURING THE OXIDATION OF MALATE PLUS L-CYSTEINESULFINATE BETWEEN THE INTRA- AND THE EXTRAMITOCHONDRIAL SPACE

The reaction mixture contained 125 mM KCl, 10 mM Tris-HCl, 5 mM P_i, 7.5 mM malate, 7.5 mM L-cysteinesulfinate, FCCP or ADP at the concentrations indicated, [^{14}C]sucrose, 4% dextran and 2.9 mg protein. The final pH was as indicated. Final volume: 1.0 ml. Temperature: 25°C. After 50 s incubation the mitochondria were separated by centrifugal filtration.

Malate + L-cysteinesulfinate	FCCP or ADP	pH	Aspartate (nmol/mg protein)	
			In	Out
—		7.4	0.7	0
+	FCCP (0.25 μM)	7.4	0.8	44.4
+	FCCP (0.5 μM)	7.4	0.7	45.2
+	FCCP (5.0 μM)	7.4	0.8	42.5
+	ADP (2.0 mM)	7.4	0.7	41.7
+	FCCP (0.25 μM)	6.4	0.7	27.4
+	FCCP (0.25 μM)	8.0	0.6	57.3

trates the dependence of the oxidation of malate and of malate plus L-cysteinesulfinate on the external pH. It can be seen that, whereas the oxidation of malate is pH independent, the malate plus L-cysteinesulfinate oxidation progressively decreases on lowering the pH of the suspending medium. The data of Fig. 2B also show that P_i is not required for the oxidation of malate plus L-cysteinesulfinate, in contrast to what was reported for the oxidation of malate plus glutamate [33].

Table I reports the amounts of aspartate present in the intra- and the extramitochondrial spaces after 50 s incubation of the mitochondria with malate and L-cysteinesulfinate. In the controls, where malate and L-cysteinesulfinate were omitted from the reaction medium, the mitochondria contain 0.6—0.8 nmol aspartate/mg protein and there is no aspartate in the suspending medium. The data of Table I show that during the oxidation of malate plus L-cysteinesulfinate all the aspartate formed is exported into the extramitochondrial space. It should be noted that approximately the same amount of aspartate is produced in the presence of ADP or increasing concentrations of FCCP. Furthermore, the formation of aspartate is considerably decreased on lowering the external pH.

Specificity of the activation of malate oxidation by L-cysteinesulfinate

The specificity of the activation of malate oxidation by L-cysteinesulfinate was investigated by studying the ability of the cysteinesulfinate analogues homocysteinesulfinate, cysteate and homocysteate to increase the rate of malate oxidation. These analogues have been found to be good substrates of the mitochondrial aspartate aminotransferase (Federici, G., unpublished results). Their effect on the respiration should therefore reflect their permeability to the mitochondria. Data not shown indicate that homocysteinesulfinate, cysteate and homocysteate, even at a concentration of 10 mM, are unable to activate the oxidation of malate. In contrast, L-cysteinesulfinate, already at a concentration of 0.75 mM, gives a value slightly higher than the half maximal

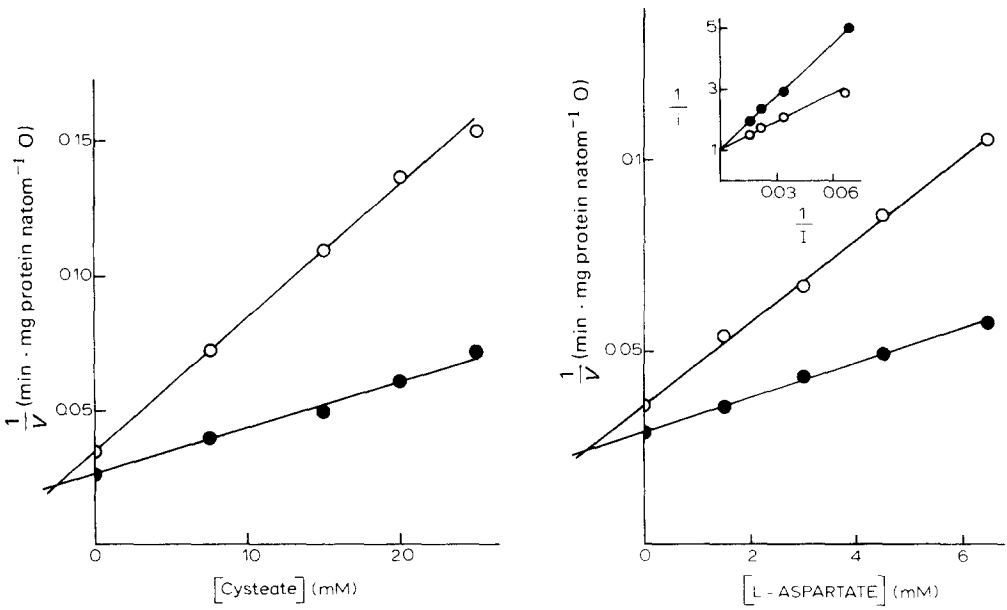

Fig. 3. Kinetic analysis of the inhibition of the L-cysteinesulfinate-induced respiration by cysteate. The incubation mixture contained 300 mM sucrose, 10 mM Tris-HCl, pH 7.2, 5 mM P_i, 1 μM FCCP, 7.5 mM malate and 3.6 mg mitochondrial protein. Cysteate, at the concentrations indicated, and 1.9 mM (○) or 5 mM (●) L-cysteinesulfinate were added. The values shown, representing the L-cysteinesulfinate-induced respiration, were obtained by subtracting from the experimental data the rate of respiration observed in the presence of malate alone.

Fig. 4. Kinetic analysis of the inhibition of the L-cysteinesulfinate-induced respiration by aspartate. Experimental conditions as in Fig. 3, except that L-aspartate at the concentrations indicated and 2.5 mM (○) or 6 mM (●) L-cysteinesulfinate were added. Mitochondrial protein was 2.9 mg. The values shown were obtained as in Fig. 3. The insert is a replot of the data, as the reciprocal of the fractional inhibition (i) versus $1/I$, where $i = 1 - (V_i/V)$, I = the concentration of aspartate, and V_i and V are the rates of oxidation in the presence of and absence of the inhibitor.

stimulation of malate oxidation obtained with L-cysteinesulfinate. Furthermore, 10 mM homocysteinesulfinate and homocysteate have no inhibitory effect on the L-cysteinesulfinate-induced respiration, whereas 10 mM cysteate causes a significant inhibition. This is observed when cysteate is added either before or after 0.75 mM L-cysteinesulfinate, and is completely released by the addition of 10 mM L-cysteinesulfinate.

The inhibition of the L-cysteinesulfinate-induced respiration by cysteate was analysed in the presence of different concentrations of L-cysteinesulfinate and/or cysteate. The data illustrated in Fig. 3 as Dixon plot show that cysteate inhibits the L-cysteinesulfinate-induced oxidation in a competitive manner with a K_i of 2.5 mM.

Mutual inhibition between L-cysteinesulfinate and L-aspartate

2.5 mM L-aspartate inhibits the oxidation of malate plus L-cysteinesulfinate when it is added either before or after 0.75 mM L-cysteinesulfinate (Fig. 1, Expts. 4—6). The effect is stereospecific since D-aspartate, D-cysteinesulfinate and D-glutamate, even at a concentration of 10 mM, do not cause any inhibi-

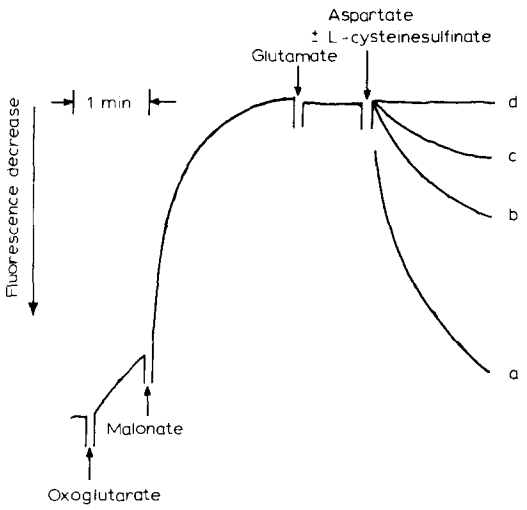

Fig. 5. Effect of L-cysteinesulfinate on the oxidation of intramitochondrial NAD(P)H by aspartate. Mitochondria (3.2 mg protein) were suspended in 1 ml of a medium containing 125 mM KCl, 10 mM Tris-HCl, pH 7.4, 5 mM P_i and 1 μM FCCP at 25°C. After 3 min 2 μg antimycin A was added, followed 1 min later by 1 mM oxoglutarate. The other additions indicated in the figure were made at the following concentrations: malonate, 1 mM; L-glutamate, 1 mM; L-aspartate, 1 mM; L-cysteinesulfinate, 5 mM in (b), 10 mM in (c) and 20 mM in (d). Where present, L-cysteinesulfinate was added simultaneously with aspartate. Pyridine nucleotide oxidation-reduction changes were followed fluorimetrically. The changes obtained before the addition of aspartate and L-cysteinesulfinate are shown by a single trace, since they were virtually identical in a—d. The interruptions of the trace are due to the opening of the cuvette container for the additions.

tion of the L-cysteinesulfinate-induced respiration (not shown).

The inhibitory effect of L-aspartate was further studied by changing the substrate and/or the inhibitor concentration. The results, reported as Dixon plot (Fig. 4), indicate that the inhibition is competitive. The completely competitive type of inhibition is demonstrated by a replot of the data, illustrated in the insert, since lines intersecting the ordinate very close to unity are obtained (see Ref. 34). A K_i = 1.1 mM for aspartate is evaluated.

Fig. 5 shows the effect of L-cysteinesulfinate on the influx of aspartate, measured fluorimetrically. After reduction of intramitochondrial $NAD(P)^+$ by oxoglutarate plus malonate and the addition of glutamate, the further addition of aspartate causes an oxidation of NAD(P)H, which reflects the formation of intramitochondrial oxaloacetate produced by transamination between aspartate and oxoglutarate [35,36]. Increasing concentrations of L-cysteinesulfinate, added simultaneously with aspartate, progressively decrease the rate of aspartate uptake. At 2.5 mM L-cysteinesulfinate (not shown) the degree of inhibition decreases from 80% with 0.25 mM aspartate to 3% with 10 mM aspartate.

Exchange between externally added L-cysteinesulfinate and intramitochondrial aspartate or glutamate

Mitochondria were loaded with various labelled metabolites, and the ability of externally added L-cysteinesulfinate to exchange with the intramitochon-

TABLE II

EXCHANGE OF INTRAMITOCHONDRIAL ASPARTATE OR GLUTAMATE WITH EXTRAMITOCHONDRIAL ANIONS

[^{3}H]Aspartate-loaded mitochondria or [^{3}H]glutamate-loaded mitochondria were incubated at 20°C for 2 min in 1.0 ml medium consisting of 130 mM KCl, 20 mM Mops, pH 7.2, 2 μg rotenone, 1 mM MalNEt, 2 mM aminooxyacetate and, except in the controls, the compounds indicated. Mitochondrial protein ranged from 2.5 to 3.2 mg protein. The data reported represent the mean of duplicate values of 3—8 experiments. Other conditions as indicated in Materials and Methods.

Additions (2 mM)	% exchange	
	Aspartate	Glutamate
L-Cysteinesulfinate	65	50
L-Glutamate	59	64
L-*allo*-γ-Hydroxyglutamate	54	58
L-Aspartate	78	55
Meso-Diaminosuccinate	45	33

drial labelled anions was tested. In experiments not shown it was found that 10 mM L-cysteinesulfinate does not cause any significant exchange with internal P_i, malate, malonate, citrate, oxoglutarate, pyruvate or ADP, indicating that the carrier systems for these substrates are not able to transport L-cysteinesulfinate.

In Table II, the ability of intramitochondrial aspartate and glutamate to exchange with L-cysteinesulfinate and other compounds, in the presence of MalNEt and aminooxyacetate, is shown. Externally added L-cysteinesulfinate (2 mM) induces a considerable efflux of intramitochondrial aspartate. Also glutamate [20], L-*allo*-γ-hydroxyglutamate [36] and *meso*-diaminosuccinate cause a significant decrease of internal aspartate. In contrast, all the other compounds tested, including aminoadipate, aminopimelate, *N*-acetylglutamate, *N*-benzoylaspartate, asparagine, glutamine, cysteine, sulfate, P_i, ADP, malate, tartrate and citrate, do not cause efflux of aspartate. It is interesting that the cysteinesulfinate analogues homocysteinesulfinate, cysteate, homocysteate, taurine and ipotaurine, as well as the D-stereoisomers of cysteinesulfinate, aspartate and glutamate do not exchange with intramitochondrial aspartate. At 10 mM external anion, a high degree of exchange (85—90%) is obtained with L-cysteinesulfinate, L-glutamate, L-*allo*-γ-hydroxyglutamate, L-aspartate, and *meso*-diaminosuccinate, and some activity is observed only with cysteate, aminoadipate, D-cysteinesulfinate, D-aspartate and D-glutamate (15—26%). Table II also shows that L-cysteinesulfinate is able to exchange with internal glutamate, the other potential product of the reaction of L-cysteinesulfinate with the aspartate aminotransferase. Significant exchange at 2 mM anion is given only by L-cysteinesulfinate, L-glutamate, L-*allo*-γ-hydroxy-glutamate, L-aspartate and *meso*-diaminosuccinate, indicating that the efflux of glutamate exhibits the same specificity as aspartate efflux. In three experiments, where L-cysteinesulfinate, homocysteinesulfinate, cysteate, asparagine, glutamine, taurine, ipotaurine, cysteine and citrate had been added externally, glutamate and aspartate were also assayed enzymatically. The results obtained from these experiments were in good agreement with the radioactive data.

Sensitivity of L-cysteinesulfinate transport to inhibitors and substrates

The exchange of L-cysteinesulfinate with intramitochondrial [^{3}H]glutamate or [^{3}H]aspartate was tested for sensitivity to inhibitors of various anion-transporting systems in mitochondria. Table III. Expt. 1, shows that the L-cysteinesulfinate-aspartate exchange is inhibited by glisoxepide, the only known inhibitor of the glutamate-aspartate carrier [37,38]. In contrast, it is not affected (data not reported) by MalNEt (1 mM), mersalyl (0.25 mM), butylmalonate (20 mM), benzene-1,2,3-tricarboxylate (10 mM), phthalonate (10 mM), bathophenanthroline (0.1 mM), carboxyatractyloside (0.1 mM) and α-cyanocynnamate (0.4 mM), which inhibit other transport systems in mitochondria. As indicated in Table III, the percent inhibition by glisoxepide of the L-cysteinesulfinate-aspartate exchange is approximately the same as for the aspartate-aspartate and the glutamate-aspartate exchagnes.

The inhibition by 5 mM glisoxepide is also observed on the uptake of 1 mM L-[^{3}H]cysteinesulfinate by aspartate-loaded mitochondria (Table III, Expt. 2). Furthermore, the L-cysteinesulfinate-aspartate exchange, measured as uptake of 1 mM labelled L-cysteinesulfinate, is inhibited by 10 mM externally added L-aspartate, L-glutamate, L-*allo*-γ-hydroxyglutamate and *meso*-diaminosuccinate. In contrast, the addition of homocysteinesulfinate, *N*-benzoylaspartate, asparagine, citrate and (not shown) P_i and malate has no effect. Table III, Expt. 2, also shows that the uptake of [^{3}H]aspartate is strongly inhibited by

TABLE III

THE INHIBITION OF THE L-CYSTEINESULFINATE-ASPARTATE EXCHANGE BY INHIBITORS AND SUBSTRATES OF THE GLUTAMATE-ASPARTATE CARRIER

The reaction mixture contained 125 mM KCl, 20 mM Mops, pH 7.2, 2 μg rotenone, 2 mM aminooxyacetate, and in Expt. 1 [^{3}H]asparate-loaded mitochondria (2.6 mg protein), in Expt. 2 aspartate-loaded mitochondria (3.4 mg protein). After 2 min the exchange reaction was initiated by the addition of 1 mM L-cysteinesulfinate, glutamate or aspartate in Expt. 1, and 1 mM L-[^{3}H]cysteinesulfinate or [^{3}H]aspartate in Expt. 2. Where indicated, 5 mM glisoxepide was included in the reaction mixture. All the other compounds tested were added simultaneously with L-[^{3}H]cysteinesulfinate or [^{3}H]aspartate at the concentrations of 10 mM. Final volume: 1 ml. Temperature: 20°C. After 1 min incubation the mitochondria were separated by centrifugal filtration.

External anion	Inhibitor	% inhibition
Expt. 1		
L-Cysteinesulfinate	Glisoxepide	65
L-Aspartate	Glisoxepide	54
L-Glutamate	Glisoxepide	61
Expt. 2		
L-[^{3}H]Cysteinesulfinate	Glisoxepide	52
	L-Aspartate	85
	L-Glutamate	84
	L-*allo*-γ-Hydroxyglutamate	68
	Meso-Diaminosuccinate	69
	Homocysteinesulfinate	7
	N-Benzoylaspartate	5
	Asparagine	4
	Citrate	7
	D-Aspartate	24
	D-Glutamate	12
L-[^{3}H]Aspartate	L-Cysteinesulfinate	82
	D-Cysteinesulfinate	15

L-cysteinesulfinate, The D-forms of cysteinesulfinate, aspartate and glutamate are much less effective. Results similar to those presented in Table III were also found using glutamate-loaded mitochondria.

Proton translocation accompanying the L-cysteinesulfinate-glutamate exchange

The electrical nature of the L-cysteinesulfinate-glutamate and L-cysteinesulfinate-aspartate exchanges has been investigated by measuring the movement of H^+ across the mitochondrial membrane.

The addition of L-cysteinesulfinate to glutamate-loaded mitochondria causes an acidification of the suspending medium (Fig. 6, trace a). The external acidification is strongly reduced by the inclusion of the transaminase inhibitor aminooxyacetate in the reaction medium (trace b). This result suggests that part of the H^+ released from the mitochondria is translocated outwards together with sulfite and pyruvate, the products of the transamination of L-cysteinesulfinate, which are known to be transported in an electroneutral H^+-compensated manner [39—41]. This explanation is supported by the finding that the acidification of the external medium is diminished by α-cyanocynnamate, an inhibitor of the pyruvate transport [42] (trace c), and stimulated by oxoglutarate (trace d). Also in the presence of oxoglutarate, aminooxyacetate

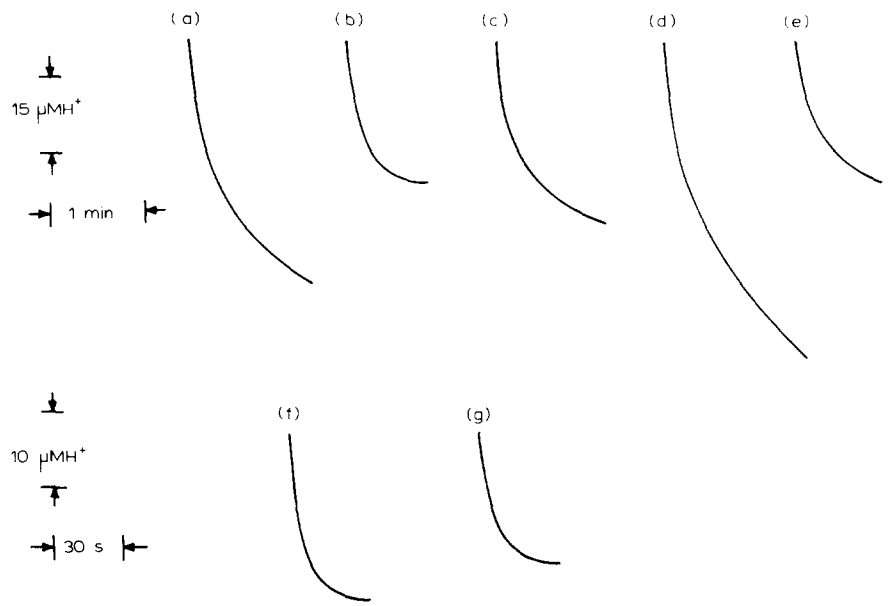

Fig. 6. pH changes of the external medium caused by the addition of L-cysteinesulfinate to glutamate-loaded mitochondria. The reaction mixture contained 150 mM KCl, 2 mM (a—e) or 1 mM (f and g) Tris-HCl, pH 7.6, 2 μg rotenone, 1 mM MalNEt and glutamate-loaded mitochondria (6.7 mg protein in a—e, or 6.3 mg protein in f and g). Additions: 3 mM aminooxyacetate (b, f, g), 0.4 mM α-cyanocynnamate (c), 1 mM oxoglutarate plus 1 mM malonate (d), and 1 mM oxoglutarate, 1 mM malonate and 3 mM aminooxyacetate (e). After 1.5 min the reaction was started by the addition of 5 mM L-cysteinesulfinate (a—f) or 5 mM L-aspartate (g). Final volume: 1 ml. Temperature: 25°C. A downward deflection of the trace indicates acidification of the extramitochondrial space. A second addition of L-cysteinesulfinate or L-aspartate did not cause any significant pH change.

reduces the L-cysteinesulfinate-induced H^+ efflux to the same extent as in its absence (cf. traces e and b). Furthermore, in experiments not shown it was found that the external acidification caused by L-cysteinesulfinate in the absence of aminooxyacetate is accompanied by the appearance of pyruvate and sulfite in the suspending medium.

The specificity of the H^+ efflux caused by L-cysteinesulfinate in the presence of aminooxyacetate was examined by testing the effect of other anions. Only L-cysteinesulfinate and L-aspartate induce efflux of H^+ from mitochondria loaded with glutamate (traces f and g). L-Glutamate, homocysteinesulfinate, L-*allo*-γ-hydroxyglutamate, cysteate, homocysteate, D-aspartate and D-cysteinesulfinate do not cause any significant pH change. The H^+ efflux, induced by either L-cysteinesulfinate or L-aspartate, is not seen in the presence of the protonophore FCCP, indicating a transmembrane H^+ movement. Furthermore, no H^+ efflux is elicited by the addition of L-cysteinesulfinate to aspartate-loaded mitochondria. In a series of six experiments the extent of the pH change and of the glutamate efflux were measured to determine the stoichiometry of the reaction. Under the conditions of Fig. 6, trace f, 3.84 ± 0.32 nmol H^+/mg protein appear in the extramitochondrial medium on addition of 5 mM L-cysteinesulfinate to glutamate-loaded mitochondria. Simultaneously the intramitochondrial content of glutamate was decreased by 4.05 ± 0.27 nmol/mg protein. The ratio $\Delta H^+/\Delta$glutamate ranged from 0.7 to 1.2 with a mean of 0.9.

Stoichiometry of the L-cysteinesulfinate-glutamate and L-cysteinesulfinate-aspartate exchanges

Glutamate-loaded mitochondria or aspartate-loaded mitochondria were incubated with labelled L-cysteinesulfinate, and after 1 min incubation the metabolite distribution between the intra- and the extramitochondrial phase was measured. Table IV reports the data of a typical experiment carried out with glutamate-loaded mitochondria in the presence of increasing concentrations of L-cysteinesulfinate. It can be seen that, at every L-cysteinesulfinate

TABLE IV

STOICHIOMETRY OF THE EXCHANGE BETWEEN L-CYSTEINESULFINATE AND INTRAMITOCHONDRIAL GLUTAMATE

The reaction mixture contained 140 mM KCl, 10 mM Mops, pH 7.2, 2 μg rotenone, 1 mM MalNEt, 2 mM aminooxyacetate, L-[^{3}H]cysteinesulfinate at the concentrations indicated, 4% dextran and glutamate-loaded mitochondria. The mitochondrial protein was 4.0 mg. The final volume was 1.0 ml. Temperature: 25°C. After 1 min incubation the mitochondria were separated by centrifugal filtration. In parallel samples ^3H_2O and [^{14}C]sucrose were added to determine the total water and the sucrose-permeable space. The intramitochondrial volume was 0.82 μl/mg protein and the sucrose-permeable space 2.8 μl/mg protein.

L-[^{3}H]Cysteinesulfinate (mM)	nmol/mg protein		ΔL-Cysteinesulfinate
	ΔL-Cysteinesulfinate	ΔGlutamate	Δglutamate
0.4	1.08	1.25	0.86
1.0	1.98	2.20	0.90
1.6	3.10	2.88	1.07
2.5	3.23	3.40	0.95
4.0	3.75	3.64	1.03

concentration used, the amount of glutamate leaving the mitochondria is approximately equal to that of L-cysteinesulfinate taken up, with a stoichiometry close to 1.

The stoichiometry of the L-cysteinesulfinate-aspartate exchange was studied using aspartate-loaded mitochondria, although, in addition to 4—8 nmol of aspartate/mg protein, they still contain some glutamate (0.5—1 nmol/mg protein). It was found that, in the presence of 1.6 mM L-cysteinesulfinate, the ratio ΔL-cysteinesulfinate/Δaspartate varied from 0.9 to 1.2 in four experiments. This suggests that there is little, if any, participation of glutamate in the L-cysteinesulfinate exchange catalyzed by aspartate-loaded mitochondria.

Discussion

The metabolism of anionic substrates by mitochondria requires in most cases the existence in the inner mitochondrial membrane of specific transport systems which usually operate by a counter exchange. L-Cysteinesulfinate is known to be an active substrate for mitochondria as also indicated by the present data of respiration and aspartate production. In this paper it is shown that L-cysteinesulfinate enters the mitochondria by a specific exchange against aspartate or glutamate. No other metabolite was found to be able to exchange with it.

Externally added aspartate competes for the uptake of L-cysteinesulfinate and, vice versa, L-cysteinesulfinate competes for the influx of aspartate indicating that L-cysteinesulfinate is transported by the same system which has been previously characterized as the mitochondrial aspartate-glutamate carrier [20,33,35—37,43]. Further evidence for this conclusion is given by the inhibition of L-cysteinesulfinate uptake by glisoxepide, the only known inhibitor of of the aspartate-glutamate carrier, and by L-glutamate, L-*allo*-γ-hydroxyglutamate and *meso*-diaminosuccinate, which are substrates of this carrier.

The use of the aspartate-glutamate carrier by L-cysteinesulfinate is not surprising in view of the structural similarities between L-cysteinesulfinate and aspartate. The replacement of $-CO_2^-$ by $-SO_2^-$ does not change the configuration of the molecule, allowing L-cysteinesulfinate to bind to the same site of the carrier. There is a strong difference in the pK of the two γ acid groups (3.7 for aspartate and 2.4 for L-cysteinesulfinate), which, however, at neutral pH has little consequence on the amount of anion present. In cysteate the more bulkier $-SO_3^-$ group permits binding to occur to the same site as aspartate although with lower affinity, but makes this substrate unable to be transported at an appreciable rate.

In further agreement with the similarity between aspartate and L-cysteinesulfinate is the finding that both, L-cysteinesulfinate and aspartate, apparently are transported in the anionic form. This is demonstrated by the lack of H^+ movement accompanying the L-cysteine sulfinate-aspartate exchange, in contrast to the translocation of H^+ observed during the L-cysteinesulfinate-glutamate exchange. It is remarkable that the structural analogue of glutamate, homocysteinesulfinate, is not transported by the same carrier. This may be due to the difference in the pK of the two δ acid groups (4.3 for glutamate and 2.6 for homocysteinesulfinate), since it has been assumed that glutamate is trans-

ported in an electroneutral manner in contrast to aspartate [20]. Since the proportion of non-ionized homocysteinesulfinate is significantly lower than that of glutamate at physiological pH, the inability of homocysteinesulfinate to be transported may indicate that the undissociated form is required for the glutamate-binding site and, therefore, this cannot be matched by the much less protonated homocysteinesulfinate. This would be at variance with the suggestion by LaNoue and coworkers, based on studies of pH dependence, that in the glutamate-protein complex the H^+ is associated to the protein rather than to the glutamate [36,44]. The inhibition of the transport activity by low pH, which we have also observed using the malate plus L-cysteinesulfinate system (Fig. 2B), may be due to an inactivation of the carrier.

Under physiological conditions it is very improbable that the electrogenic exchange between L-cysteinesulfinate and internal glutamate occurs against the electrochemical gradient created by respiration. The electroneutral exchange between L-cysteinesulfinate and internal aspartate, on the other hand, is likely to occur. It can be suggested that the L-cysteinesulfinate-aspartate exchange competes with the glutamate-aspartate exchange influencing the overall activity of the malate-aspartate shuttle, but to what extent this may occur remains to be investigated.

Finally, the different electrical nature of the glutamate-aspartate and L-cysteinesulfinate-aspartate exchanges gives an explanation of the greater ability of L-cysteinesulfinate with respect to glutamate to remove the intramitochondrial oxaloacetate in the presence of dinitrophenol [4]. Thus the L-cysteinesulfinate-aspartate exchange is not affected by the deenergization of the membrane caused by an uncoupler (Fig. 2A and Table I).

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