The Mechanism of the Inhibition of the Mitochondrial Pyruvate Transporter by α -Cyanocinnamate Derivatives

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Pyruvate transport into rat liver mitochondria is inhibited by a variety of thiol reagents. α -Cyanocinnamate and its derivatives, specific, potent and reversible inhibitors of pyruvate transport, react reversibly with mercaptoethanol and cysteine to form addition products. It is concluded that these inhibitors react with an essential thiol group on the pyruvate carrier.

α -Cyano-4-hydroxycinnamate is a very powerful inhibitor of the mitochondrial transport of pyruvate (Halestrap & Denton, 1974; Halestrap, 1975). Kinetic studies of the carrier involved in this transport process indicate that inhibition is both non-competitive and reversible, which may indicate that inhibition involves the reversible formation of a covalently bound inhibitor-carrier complex (Halestrap, 1975). Other workers (Papa *et al.*, 1971; Papa & Paradies, 1974; Paradies & Papa, 1975) have shown that the carrier is sensitive to the thiol-group reagents mersalyl and *N*-ethylmaleimide. In this present paper I confirm and extend their results and show that α -cyanocinnamate and its derivatives are also powerful but readily reversible thiol-group reagents. The reaction involves addition of the thiol group across the double bond between the α - and β -carbon atoms of α -cyanocinnamate, as depicted in reaction (1), and may be followed by observing the resultant change in the u.v. spectrum:

Dorset, U.K., fluorodinitrobenzene and sodium mersalyl were from Sigma (London) Chemical Co., London S.W.6, U.K., and α -bromophenylacetic acid was from Aldrich Chemical Co., Wembley, Middx., U.K. The sources of all other chemicals and radiochemicals are those given in Halestrap (1975).

Results and Discussion

The effect of a variety of thiol-group reagents on the uptake of pyruvate into mitochondria is reported in Table 1. *N*-Ethylmaleimide and mersalyl inhibited pyruvate uptake at 22°C, as described by others (Papa *et al.*, 1971; Papa & Paradies, 1974; Paradies & Papa, 1975), without having any effect either on the intramitochondrial space or on the accumulation of acetate (measured as described by Halestrap & Denton, 1974). At 6°C, however, these reagents were not effective inhibitors, implying that inhibition involves slow covalent-bond formation. This observation may explain some of the anomalous results

Materials and Methods

Preparation of rat liver mitochondria and measurement of their accumulation of pyruvate driven by a pH gradient were performed as described previously (Halestrap, 1975). U.v.-absorption spectra and extinction changes were obtained by using a Unicam SP.1800 split-beam recording spectrophotometer. *N*-Ethylmaleimide, 5,5'-dithiobis-(2-nitrobenzoic acid), iodoacetic acid and bromopyruvic acid were obtained from BDH Chemicals, Poole,

obtained by Papa & Paradies (1974) on the inhibition of pyruvate and acetoacetate exchange reactions by thiol-group reagents. A large variety of thiol-group reagents are effective inhibitors of mitochondrial pyruvate transport, but none is as effective as α -cyano-4-hydroxycinnamate and its analogues (Table 1). Even bromopyruvate, which was the most effective of the other reagents used, required pre-incubation with mitochondria before inhibition became apparent. In contrast, α -cyano-4-hydroxycinnamate inhibits totally and rapidly at both 6°C

Table 1. Inhibition of mitochondrial pyruvate transport by thiol-group reagents

Rat liver mitochondria (about 8 mg of protein) were preincubated in 1 ml of medium [0.125M-KCl/0.02M-2-(N-2-hydroxyethylpiperazin-*N'*-yl)ethanesulphonic acid, pH 6.8] containing 10 μ M-rotenone, 10 μ M-antimycin A, [6,6-³H]sucrose (1 μ Ci/ml) and other additions as indicated for 5 min at the temperature shown. [2-¹⁴C]Pyruvate (0.15 mM and 0.6 μ Ci/ μ mol) was added and incubation continued for 1 min before centrifugation at 15000g_{av} for 1 min to sediment mitochondria. Analysis of the mitochondrial pellet for intramitochondrial pyruvate was performed as described previously (Halestrap & Denton, 1974). All results are given as the means $\pm$ S.E.M. of four separate observations. The statistical significance of the experimental values when compared with control values was calculated by Student's *t* test: **P* < 0.01.

Additions	Pyruvate content of mitochondria (nmol/mg of mitochondrial protein)	
	6°C	22°C
None	0.58 $\pm$ 0.04	0.72 $\pm$ 0.04
0.1 mM- α -Cyano-4-hydroxycinnamate	<0.01	0.04 $\pm$ 0.02*
2.0 mM-Iodoacetate	0.62 $\pm$ 0.01	0.52 $\pm$ 0.03*
2.0 mM-Iodoacetate + 0.1 mM- α -cyano-4-hydroxycinnamate	<0.01	0.07 $\pm$ 0.04*
2.0 mM- <i>N</i> -Ethylmaleimide	0.46 $\pm$ 0.02	0.25 $\pm$ 0.02*
1.0 mM-Sodium mersalyl	0.60 $\pm$ 0.03	0.31 $\pm$ 0.02*
1.0 mM-5,5'-Dithiobis (2-nitrobenzoate)	—	0.48 $\pm$ 0.03*
2.0 mM- α -Bromophenylacetate	0.33 $\pm$ 0.01*	0.41 $\pm$ 0.01*
2.0 mM-Bromopyruvate	0.53 $\pm$ 0.05	0.07 $\pm$ 0.02*
0.5 mM-Fluorodinitrobenzene	0.39 $\pm$ 0.03*	0.32 $\pm$ 0.03*
0.5 mM-Fluorodinitrobenzene + 0.1 mM- α -cyano-4-hydroxycinnamate	<0.01	—

Fig. 1. Spectral changes associated with the addition of mercaptoethanol to α -cyanocinnamate analogues

(a) shows the u.v.-absorption spectra of 50 μ M-cyanocinnamate in 100 mM-Tris/HCl buffer, pH 7.4, at 30°C after equilibration with: no addition (i); 2 mM-mercaptoethanol (ii); 10 mM-mercaptoethanol (iii). The broken line shows the spectrum of (iii) re-run after exposure to 20 mM-H₂O₂ for 1 h. In (b) the extinctions of various α -cyanocinnamate analogues at 50 μ M were measured in 100 mM-Tris/HCl buffer, pH 7.4, at the absorption maxima given in parentheses: (i) α -cyano-5-phenylpenta-2,4-dienoate (328 nm); (ii) α -cyano- β -(1-phenylindol-3-yl)acrylate (370 nm); (iii) α -cyano-4-hydroxycinnamate (325 nm); (iv) α -cyanocinnamate (285 nm); (v) and (vi) α -cyano-3-hydroxycinnamate (300 nm). Additions were made as indicated: A, 15 μ mol of mercaptoethanol; B, 70 μ mol of mercaptoethanol; C, 60 μ mol of H₂O₂; D, 70 μ mol of mercaptoethanol. In (vi) α -cyanocinnamate was treated with 15 μ mol of mercaptoethanol as in (iv), before addition of H₂O₂. The temperature was 30°C and the volume of the cuvette 3 ml.

and 30°C (Halestrap, 1975). Inhibition was also observed with fluorodinitrobenzene, a reagent used for labelling amino groups, which might be taken to indicate the involvement of amino groups in the transport of pyruvate. However, this reagent is known to react with other groups, including essential thiol

groups (Hirs, 1967). None of the thiol-group reagents used in the experiments of Table 1 prevented complete inhibition of the transport of pyruvate by α -cyano-4-hydroxycinnamate.

Fig. 1(a) shows the u.v. spectrum of α -cyano-cinnamate, which has an absorption maximum at

295 nm with a molar extinction coefficient of $25 \times 10^3 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$. Addition of mercaptoethanol to a solution of α -cyanocinnamate produces a rapid fall in the E_{295} (Fig. 1*b*), and the new u.v. spectrum is without an absorption maximum at 295 nm (Fig. 1*a*). Removal of the mercaptoethanol by oxidation with H_2O_2 regenerates the original spectrum (Figs. 1*a* and 1*b*). These spectral changes suggest that mercaptoethanol adds across the double bond of α -cyanocinnamate, as depicted in reaction (1). The loss of conjugation that results causes the loss of the absorption maximum at 295 nm. Identical spectral changes can be induced by addition of cysteine or other primary thiols, but not by primary amines or any of the naturally occurring amino acids, other than cysteine. Similar reactions have been studied by Holmes (1975) for a wide range of compounds of the general formula:

The reactions are freely reversible, the rates of the forward and reverse reactions depending on the nature of A, B, R, X and Y. In Fig. 1*b* it is shown that several analogues of α -cyanocinnamate that are powerful inhibitors of mitochondrial transport (Halestrap, 1975) also react with mercaptoethanol. α -Fluorocinnamate and cinnamate are not reactive towards mercaptoethanol, however, nor are they inhibitors of mitochondrial pyruvate transport.

Inhibition of mitochondrial pyruvate transport by α -cyano-4-hydroxycinnamate and its analogues is non-competitive, reversible and dependent on the nature of the hydrophobic moiety of the molecule (Halestrap, 1975; Halestrap & Denton, 1975). This pattern of inhibition would be expected if an essential thiol group is attacked by the inhibitor in an analogous fashion to the model reaction described above. The binding of the aromatic moiety of the inhibitor to a hydrophobic region of the carrier protein would bring the thiol group and active double bond into close proximity, effectively giving extremely high local concentrations of inhibitor. This would allow rapid addition of the thiol group across the double bond of the inhibitor and drive the equilibrium towards complete inhibition. A comparison of the reactivity of α -cyano-cinnamate analogues towards mercaptoethanol and their potency as inhibitors of pyruvate transport suggests that the latter property is affected more by the binding of the inhibitor to hydrophobic regions of the protein, to create localized high concentrations, than by the intrinsic reactivity of

the double bond. Thus the rate and extent of the reaction with mercaptoethanol are greatest for α -cyanocinnamate and α -cyano-3-hydroxycinnamate, followed by α -cyano-4-hydroxycinnamate, α -cyano- β -(1-phenylindol-3-yl)acrylate and, least reactive of all, α -cyano-5-phenylpenta-2,4-dienoate (Fig. 1*b*). This complies with the general principles of reactivity described by Holmes (1975), but does not show any close relationship to the potency of these compounds as inhibitors of mitochondrial pyruvate transport. Here α -cyano-5-(1-phenylindol-3-yl)-acrylate is the most potent inhibitor (K_i about $0.05 \mu\text{M}$), followed by α -cyanocinnamate and α -cyano-5-phenylpenta-2,4-dienoate (K_i values about $0.2 \mu\text{M}$), and α -cyano-3-hydroxycinnamate and α -cyano-4-hydroxycinnamate (K_i values about $1.5 \mu\text{M}$) (Halestrap, 1975).

To prove conclusively that α -cyanocinnamate and its analogues do inhibit mitochondrial pyruvate transport by attacking a specific thiol group would require demonstration of the formation of a thiol- α -cyanocinnamate addition product in the mitochondrial membrane. However, this reaction is freely reversible, and no way of stabilizing the addition product has been found. U.v. light and oxidizing and reducing reagents were all found to be ineffective. Nor could α -cyanocinnamate protect pyruvate transport from inhibition by other thiol-group reagents. This is to be expected if the reaction of α -cyanocinnamate is reversible, whereas that of alkylating thiol-group reagents is not. Indeed, *N*-ethylmaleimide could be shown to regenerate α -cyano-4-hydroxycinnamate from its addition product with mercaptoethanol. Thus the evidence reported here strongly suggests that α -cyanocinnamate is a very specific, potent and reversible thiol-group reagent for the mitochondrial pyruvate carrier.

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