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Characterization of the α-cyanocinnamate binding site in rat heart mitochondria and in submitochondrial particles

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The effect of pH and substrates on the binding of radiolabelled α-cyanocinnamate to mitochondria and submitochondrial particles has been investigated. It has been found that the binding is strongly influenced by the pH of the medium (it decreases on increasing the pH of the medium). The inhibition of pyruvate oxidation by this inhibitor follows the same pH dependence. The pH affects only the affinity of the α-cyanocinnamate binding site without changing their total number. A similar pH dependence has been found in inside-out submitochondrial particles where the binding sites are directly accessible. The quantitative parameters of the binding of α-cyanocinnamate in submitochondrial particles have been determined. The binding can be prevented or displaced by pyruvate and other substrates of the carrier. The turnover number for pyruvate transport in rat-heart mitochondria has been determined.

Introduction

The transport of pyruvate into mitochondria is mediated by a specific transporting system which brings about both a pyruvate/pyruvate exchange and a net movement of the anion across the inner membrane, the latter process being accompanied by the cotransport of H^+ (or the countertransport of hydroxyl ions) [1–3].

Although several aspects of this carrier, such as substrate specificity and kinetic properties, have been well elucidated [4–8], little is known about its molecular nature.

The use of specific inhibitors has been very useful to define several molecular properties of various mitochondrial anion transporting systems [9–12]. α-Cyanocinnamate is a very powerful and specific inhibitor of the transport of pyruvate across the mitochondrial membrane. It has been widely used to characterize the mitochondrial pyruvate translocator [13–14], as well as for the study of those metabolic pathways which involve the mitochondrial transport of this ketoacid [15–16].

Further insight into the molecular mechanism of the pyruvate translocator in mitochondria may be achieved by following the binding of α-cyanocinnamate to the mitochondrial membrane. Recently, by using radiolabelled α-cyanocinnamate, several aspects of the binding of this inhibitor to rat-heart mitochondrial membrane have been investigated [17]. In the present work, further studies on the binding of radiolabelled α-cyanocinnamate to mitochondria have been carried out. Particularly the effect of pH and substrates on the binding parameters of α-cyanocinnamate has been investigated and correlated to the inhibition of pyruvate transport. These binding experiments have been extended to inside-out submitochondrial particles.

The mechanism by which α-cyanocinnamate inhibits the transport of pyruvate into mitochon-

dria involves the binding of the inhibitor to SH groups which are essential for the carrier activity [18]. Thus the characterization of the α-cyanocinnamate binding site in mitochondria and in submitochondrial particles may give important information on the role of the thiol groups in the functioning of the pyruvate carrier. In addition, the characterization of the α-cyanocinnamate binding site may represent an important preliminary step in the isolation and identification of the pyruvate carrier protein.

Materials and Methods

Biological preparations. Rat heart mitochondria were prepared according to Ref. 19. 0.25 M sucrose was used for homogenization and washing. The mitochondria used in the present work showed high respiratory control ratios above 7 and the ADP/oxygen ratios observed with pyruvate and malate as substrate ranged from 2.4 to 2.8.

Inside-out submitochondrial particles were prepared by sonication of the rat-heart mitochondria after dilution to a concentration of 10–15 mg protein/ml in 0.25 M sucrose/5 mM Tris-HCl (pH 8.5). The mitochondrial suspension was exposed to successive sonic oscillations in a Branson sonifier at 90–100 W for five periods of 30 s each, separated by a 1 min interval. The temperature of the suspension was maintained at 2–5°C. Unbroken mitochondria were removed by centrifugation at 12000 × *g*. The sonic particles were recovered from the supernatant by centrifugation at 30000 rpm (Beckman ultracentrifuge, rotor 60 Ti) for 60 min. After washing in 0.25 M sucrose/5 mM Tris-HCl buffer (pH 7.0), the final pellet was resuspended in the same medium.

The standard medium used in the measurements of respiratory activity and in the binding experiments usually contained 100 mM sucrose/50 mM KCl/20 mM Tris-HCl/1 mM $MgCl_2$/0.5 mM EDTA.

Measurements of respiration. Rates of oxygen consumption in mitochondria were measured in a thermostatically controlled oxygraph vessel with a Clark-type electrode (YSI model 53, oxygen monitor, Yellow Spring Instrument Co., Yellow Spring, OH).

Measurements of binding. The binding of α-cyanocinnamate to mitochondria was assayed as follow s(see also Ref. 17). The mitochondria (1–2 mg of protein/ml) were incubated at room temperature in the standard medium described above. The total volume was 1 ml. After 3 in of preincubation, labelled α-cyanocinnamate was added and 3 min later the reaction was stopped by rapid centrifugation of the mitochondria suspension in the cold at 15000 × *g*. The supernatant was then decanted and the pellet was rinsed with 15% (w/v) $HClO_4$. Next the binding was determined by measuring the radioactivity in the sediment and in the supernatant by liquid scintillation counting. The radioactivity of the pellet was corrected for the α-cyanocinnamate in the extramitochondrial space. The latter was determined in separate samples by the distribution of [^{14}C]sucrose.

The binding of α-cyanocinnamate to submitochondrial particles was determined as described above for the intact mitochondria, the only difference being that the particles were precipitated at 70000 × *g* for 15 min.

Mitochondrial protein were determined by the usual biuret method. α-Cyanocarboxyl [^{14}C]cinnamic acid was synthesized by Amersham International. Its specific activity was 19.2 mCi/mmol and its purity was 98%.

All other reagents were of reagent grade purity and were purchased from Sigma.

Results

The effect of pH on the binding of α-cyanocinnamate and on the inhibition of pyruvate transport in rat-heart mitochondria is reported in Fig. 1. The pyruvate transport has been studied by following the rate of pyruvate-dependent oxygen uptake in the presence of ADP (under this condition the transport of pyruvate has been shown to be a rate-limiting step for oxidation) [20]. The inhibition of pyruvate oxidation by α-cyanocinnamate exhibits a strong pH dependence. In fact, in the presence of 0.25 μM of α-cyanocinnamate, the inhibition of pyruvate oxidation, which at pH 6.6–7.2 amounted to 95%, strongly decreased on increasing the pH of the medium, dropping to around 10% at a pH value of 7.8.

The binding of α-cyanocinnamate followed the

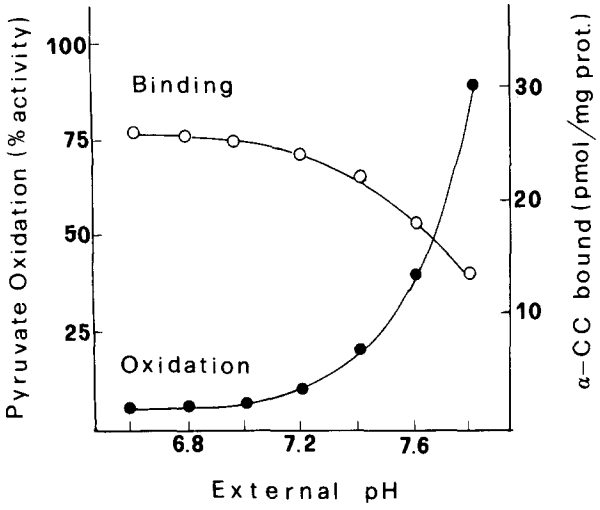

Fig. 1. The effect of pH on the binding of α-cyanocinnamate to rat-heart mitochondria and correlation to the inhibition of pyruvate oxidation. The pyruvate-dependent oxygen uptake was measured with a Clark-type electrode. Mitochondria (1 mg protein/ml) were preincubated in the standard medium described in Material and Methods, in the presence of 2 mM ADP and 2 mM P_i, at 25°C. When a steady state of oxygen consumption was obtained, 0.5 mM pyruvate was added. The rate of respiration that followed within 30 s of pyruvate addition was used for calculating the rate of pyruvate-dependent oxygen uptake. When present, α-cyanocinnamate (α-CC) 0.250 μM was added in the preincubation mixture 3 min prior the addition of pyruvate. The rate of pyruvate oxidation in the absence of the inhibitor amounted to around 220 natom oxygen per min/mg protein and it was substantially unchanged at the various pH values used. The binding of α-cyanocinnamate was determined as described in Materials and Methods. ○, α-cyanocinnamate binding; ●, percentage pyruvate oxidation.

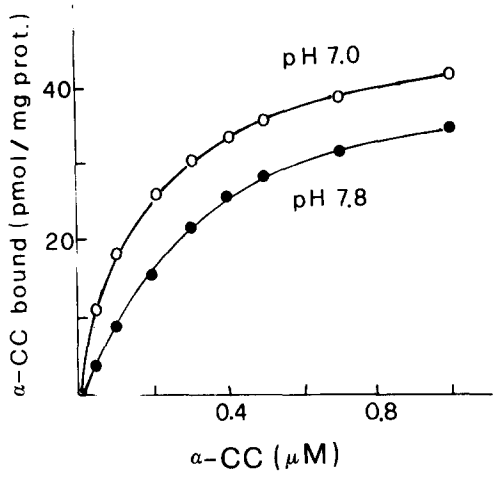

Fig. 2. The effect of pH on the binding of increasing concentration of α-cyanocinnamate (α-CC). The binding of α-cyanocinnamate was determined as described in Materials and Methods. Mitochondria (1.1 mg protein/ml) were preincubated in the standard medium described in Materials and Methods at the pH values indicated. After 3 min of preincubation, increasing concentrations of labelled inhibitor were added, and 3 min later mitochondria were separated from the medium by rapid centrifugation. ○, pH 7.0; ●, pH 7.8.

same pH dependence as that of the inhibition of pyruvate oxidation.

In order to explore better the pH dependence of the binding of α-cyanocinnamate to mitochondria, the effect of pH 7.0 and 7.8 on the binding of different concentrations of the inhibitor was investigated. The result of this experiment is reported in Fig. 2. The binding of α-cyanocinnamate reached saturation at both these pH values. However, the binding capacity was significantly lower at pH 7.8 with respect to pH 7.0. The binding data of Fig. 2 were analyzed by Scatchard plots and the results are reported in Fig. 3. It can be seen that whilst the maximal number of binding sites remains unchanged there is a significant increase in the dissociation constant at pH 7.8 as compared to pH 7.0.

Since sonic particles have their membranes reversed with respect to that of intact mitochondria, thus exposing the binding sites directly to the external medium, they may be used to study more directly the interaction of α-cyanocinnamate with the mitochondrial pyruvate translocator. Unfortunately a parallel study of pyruvate transport in these particles was not possible. However, experimental conditions very similar to those used to follow the binding in intact mitochondria were chosen to study this process in sonic particles. The binding of α-cyanocinnamate to sonic particles was analyzed by its concentration dependence in the range 0.025–1 μM of the inhibitor and at both pH 7.0 and 7.8. α-Cyanocinnamate was found to bind readily and with high affinity to sonic particles (Fig. 4). The α-cyanocinnamate binding curve was characterized by a saturation plateau and exhibited a pH dependence similar to that found in intact mitochondria. Scatchard plots of the binding data of Fig. 4 allowed the quantitative parameters of α-cyanocinnamate to sonic particles to be determined. The maximal amount of the

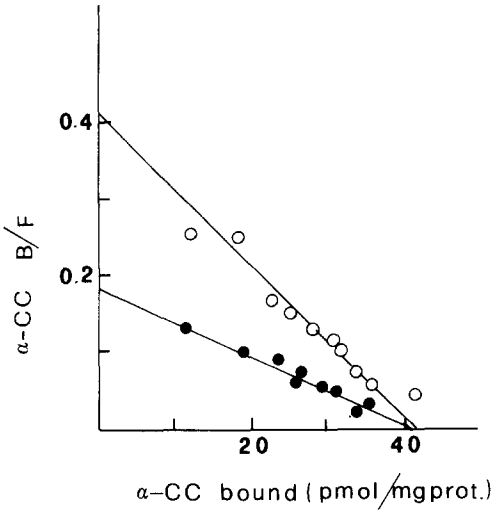

Fig. 3. Scatchard plots of α-cyanocinnamate (α-CC) binding to rat-heart mitochondria at different pH values. Data from Fig. 2.

inhibitor bound with high affinity was approx. 60 pmol/mg of protein at pH 7.0 and 25°C, a value slightly higher than that found in intact mitochondria, whereas the dissociation constant was around 0.1 μM, a value identical to that found in intact mitochondria. As found in intact mitochondria (see Fig. 4) in sonic particles, the pH affected only the dissociation constant of the α-cyanocinnamate binding sites without changing their total number.

The substrate specificity of the binding of α-cyanocinnamate in sonic particles was studied by testing the effect of different anionic substrates on their ability either to prevent or to displace the binding. The results of this experiment, reported in Table I, demonstrate that when added before the labelled inhibitor, pyruvate and other substrates of the carrier such as 2-oxobutyrate, acetoacetate and phenylpyruvate were able to considerably inhibit the binding of α-cyanocinnamate, whereas they displace bound α-cyanocinnamate when added 5 min after. The degree of inhibition and displacement seems to be related to the different affinity, exhibited by these substrates, for the pyruvate carrier in intact mito-

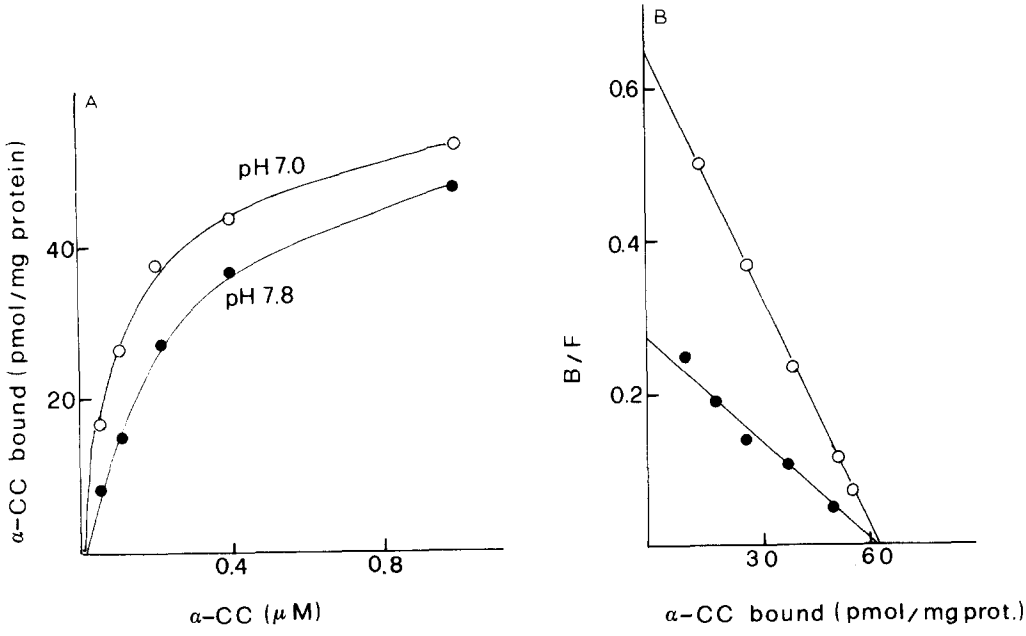

Fig. 4. The binding of α-cyanocinnamate (α-CC) to rat-heart submitochondrial particles and the effect of pH. (A). Submitochondrial particles (0.7 mg protein/ml) were preincubated in the standard medium described in Materials and Methods at 25°C. After 3 min, increasing concentrations of labelled α-cyanocinnamate were added and 3 min later the particles were separated from the medium by rapid centrifugation. The binding of α-cyanocinnamate was determined as described in Materials and Methods. (B) Scatchard plots of the binding of α-cyanocinnamate at pH 7.0 and 7.8. The number of specific binding sites = 58 ± 10 pmol per mg of protein; dissociation constant (K_d) = 0.1 ± 0.011 μM. The values are the means ± S.E. for five experiments.

TABLE I

THE EFFECT OF VARIOUS MONOCARBOXYLIC ACIDS ON THE BINDING AND ON THE RELEASE OF α-CYANOCINNAMATE IN RAT-HEART SUBMITOCHONDRIAL PARTICLES

The binding of α-cyanocinnamate was measured as described in the legend of Fig. 4. Submitochondrial particles (0.8 mg of protein/ml) were preincubated in the standard medium described in Materials and Methods. After 3 min of preincubation, α-cyanocinnamate (0.2 μM) was added and 5 min later the particles were separated from the medium by rapid centrifugation. Pyruvate and other monocarboxylic acids were added in the preincubation phase at a concentration of 2 mM. The release of bound α-cyanocinnamate was determined essentially as described above with the only exception being that pyruvate and other substrates were added 5 min after the addition of the labelled inhibitor, followed 6 min later by rapid centrifugation. The results are expressed as means ± S.E. of four separate observations.

Additions	Bound α-cyanocinnamate (pmol/mg of protein)	Inhibition (%)	Remaining bound α-cyanocinnamate (pmol/mg of protein)	Displacement (%)
Control	35.2 ± 5.3		34.5 ± 4.2	
Pyruvate	10.1 ± 1.8	71.3	12.4 ± 2.1	64
2-Oxobutyrate	15.4 ± 2.7	56.3	18.2 ± 3.8	47
Acetoacetate	18.3 ± 4.1	48.0	20.3 ± 4.0	41
Phenylpyruvate	8.9 ± 1.8	74.7	10.2 ± 2.1	70
Acetate	34.4 ± 6.1	2.0	33.8 ± 5.3	2
α-Cyanocinnamate (unlabelled)			3.2 ± 0.8	93

chondria [8]. Acetate, which is not a substrate for the carrier, did not affect either the binding or the release of α-cyanocinnamate. Bound α-cyanocinnamate could be readily and totally displaced by unlabelled α-cyanocinnamate, which speaks in favour of specific α-cyanocinnamate binding sites.

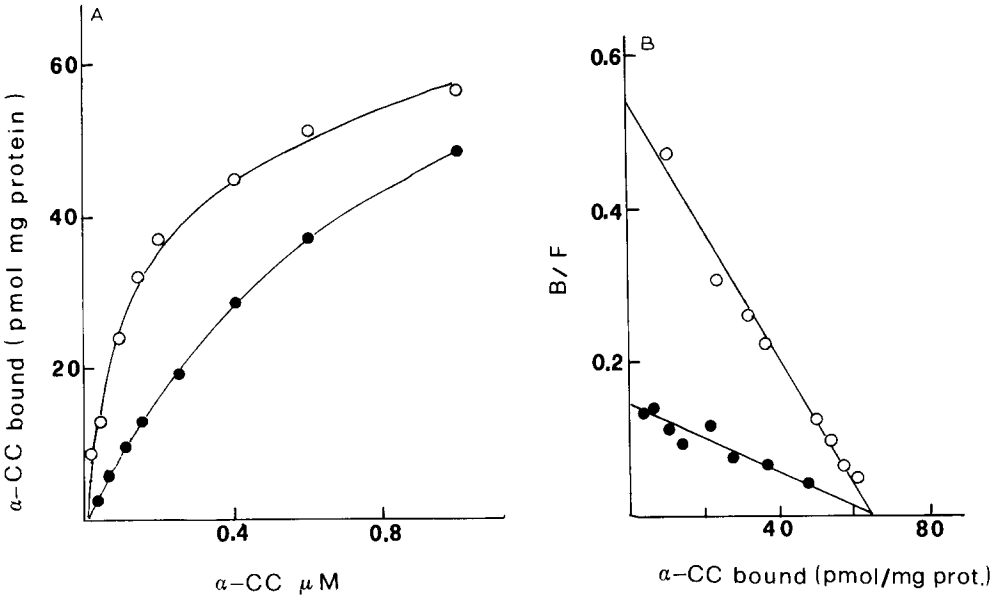

Fig. 5. The effect of pyruvate on the binding of increasing concentrations of α-cyanocinnamate (α-CC) to submitochondrial particles. (A) The binding of α-cyanocinnamate was determined as described in the legend to Fig. 4 and in Materials and Methods. Submitochondrial particles (0.6 mg protein/ml) were preincubated in the standard medium described in Materials and Methods. After 3 min of preincubation, increasing concentrations of labelled α-cyanocinnamate were added and 3 min later the particles were separated from the medium by rapid centrifugation. Pyruvate (2 mM) was added in the preincubation phase. (B) Scatchard plots of the binding of α-cyanocinnamate and the effect of pyruvate. ○, control; ●, pyruvate in the preincubation.

TABLE II

THE EFFECT OF VARIOUS MONOCARBOXYLATES ON THE RELEASE OF BOUND α-CYANOCINNAMATE IN RAT-HEART SUBMITOCHONDRIAL PARTICLES

The binding of α-cyanocinnamate was measured as described in the legends to Fig. 4 and Table I. Submitochondrial particles (0.8 mg protein/ml) were preincubated in the standard medium described in Materials and Methods. After 3 min of preincubation α-cyanocinnamate (0.2 μM) was added and 6 min later the particles were separated from the medium by rapid centrifugation. Pyruvate and other monocarboxylic acids were added 3 min after the addition of the labelled inhibitor at concentrations of 2 mM. The results are expressed as means ± S.E. of four separate observations.

Addition	Remaining bound α-cyanocinnamate (pmol/mg of protein)	Displacement (%)
None	34.5 ± 4.2	
Pyruvate	12.4 ± 2.1	64
2-Oxybutyrate	18.2 ± 3.8	47
Acetoacetate	20.3 ± 4.0	41
Phenylpyruvate	10.2 ± 2.1	70
Acetate	33.8 ± 5.3	2
α-Cyanocinnamate (unlabelled)	3.2 ± 0.8	93

The interaction of pyruvate with the α-cyanocinnamate binding site in sonic particles was better investigated in the experiment reported in Fig. 5, which shows the effect of pyruvate on the binding of increasing concentrations of labelled inhibitor. At a 2 mM concentration, pyruvate strongly decreased the binding of α-cyanocinnamate, the inhibition being higher at lower concentrations of the inhibitor. Scatchard plots of these binding data demonstrate that whilst the total number of binding sites remained unchanged, there was a strong decrease in the dissociation constant value. From the data of Fig. 5 a K_i value for inhibition by pyruvate of α-cyanocinnamate binding of about 0.5 mM was evaluated, consistent with the value for the K_m of pyruvate transport in rat-heart mitochondria [14].

Discussion

As shown in a previous paper, rat-heart mitochondria contain a specific binding site for α-cyanocinnamate which is directly involved in the inhibition of pyruvate transport [17]. In fact, it was shown that there is a close correlation between the binding of α-cyanocinnamate to mitochondria and the inhibition of pyruvate transport, both functions reaching saturation at the same titre of the inhibitor. In the present work, the binding of α-cyanocinnamate to intact mitochondria and to inside-out submitochondrial particles has been further characterized. It has been found that the binding of α-cyanocinnamate as well as the inhibition of pyruvate oxidation by this inhibitor are strongly influenced by the pH of the medium, both functions decreasing on increasing the pH. This strong pH-dependence cannot be ascribed to a decreased permeability of the inhibitor to the inner mitochondrial membrane where the α-cyanocinnamate binding sites are located [14]. In fact, the same pH dependence has been found in inside-out submitochondrial particles which are inverted framgents of the inner membrane of mitochondria, thus exhibiting the binding sites directly exposed to the pH of the medium. In addition, it has been found (results not shown) that the inhibition by α-cyanocinnamate of pyruvate oxidation at pH 7.0 can be readily and totally reversed by increasing the pH of the medium to pH 7.8. It is believed that the pH dependence of the binding of α-cyanocinnamate and the related inhibition of pyruvate utilization by this inhibitor could be attributed to an intrinsic property of the α-cyanocinnamate binding site and/or to the functioning of the carrier itself.

It has been well established that the mechanism by which α-cyanocinnamate inhibits the pyruvate carrier involves the binding of the inhibitor to SH groups which are essential for the transporting activity [18]. Interestingly enough, the pH affects only the K_d value of the binding of α-cyanocinnamate, leaving the total number unchanged, both in intact mitochondria and in submitochondrial particles (see Fig. 3 and 4). Thus the effect of pH could be attributed either directly to a change in the reactivity of the SH groups involved in the binding of the inhibitor or indirectly to a change in the neighbouring structures in which these SH groups are located within the carrier polypeptide chain. In addition, the pH dependence could be the result of a conformational change induced, for example, by changes of the charge density at the regions of the α-cyanocinnamate binding site.

It was previously shown that the substrates of the carrier strongly inhibit the specific binding of α-cyanocinnamate in intact mitochondria [17]. Similar results have been obtained for the inside-out submitochondrial particles which expose the binding sites directly to the medium. In addition, it has been found (see Fig. 5) that pyruvate inhibits in a competitive manner the binding of α-cyanocinnamate. These results support the view that pyruvate and α-cyanocinnamate share a common binding site on the carrier protein as previously suggested by Halestrap [2].

The similarity between the binding properties of α-cyanocinnamate in intact mitochondria and in submitochondrial particles indicate that the α-cyanocinnamate binding site is not altered by the sonication procedure used to prepare these vesicles. Therefore, the latter may be used to further characterize the α-cyanocinnamate binding site and also as starting material for the enrichment and isolation of the carrier molecule.

The quantitative parameters of the α-cyanocinnamate binding sites allow the calculation of the value for the turnover number of the carrier in rat-heart mitochondria. The V_{max} for net pyruvate uptake in these mitochondria, determined by the inhibitor stop technique, has been reported to be around 5 nmol/min per mg protein at 6°C [14]. This datum has been confirmed in our laboratory. At 25°C a V_{max} value of 90 nmol/min per mg protein can be calculated using the temperature coefficient value reported by Halestrap [2]. At this temperature, the number of pyruvate carrier molecules corresponds to approx. 50 pmol/mg protein (see present study and previous paper [17]). This value is nearly half of the one recently reported by other authors [20] who estimated the number of carrier molecules by indirect inhibitory measurements. Thus, an estimate of the turnover number of pyruvate carrier at 1800 min^{-1} can be derived.

Work is in progress to identify the pyruvate carrier protein by specific labelling with radiolabelled α-cyanocinnamate.

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References

1 Papa, S. and Paradies, G. (1974) Eur. J. Biochem. 49, 265–274
2 Halestrap, A.P. (1975) Biochem. J. 148, 85–96
3 Vaartjes, W.J., Geelen, M.J.H. and Van den Bergh, S.G. (1979) Biochim. Biophys. Acta 548, 38–47
4 Paradies, G. and Papa, S. (1975) FEBS Lett. 52, 149–152
5 Paradies, G. and Papa, S. (1976) FEBS Lett. 62, 318–321
6 Halestrap, A.P., Scott, R.D. and Thomas, A.P. (1980) Int. J. Biochem. 11, 97–105
7 Paradies, G. and Papa, S. (1977) Biochim. Biophys. Acta 462, 333–346
8 Paradies, G. and Papa, S. (1978) in Bioenergetics at Mitochondrial and Cellular Levles (Wojtczak, L., Lenatowicz, E. and Zborowski, J., eds.), pp. 39–77, Nencki Institute of Experimental Biology, Warsaw
9 Klingenberg, M., Appel, M., Babel, W. and Aquila, H. (1983) Eur. J. Biochem. 131, 647–654
10 Lauquin, G.J.M. and Vignais, P.V. (1976) Biochemistry 15, 2316–2332
11 Palmieri, F., Stipani, I., Quagliariello, E. and Klingenberg, M. (1972) Eur. J. Biochem. 26, 587–594
12 Fonyo, A. (1978) Journal of Bioenergetics and Biomembrane 10, 171–194
13 Halestrap, A.P. (1978) Biochem. J. 172, 377–387
14 Thomas, A.P. and Halestrap, A.P. (1981) Biochem. J. 196, 471–479
15 Mendez-Mourão, J., Halestrap, A.P., Crisp, D.M. and Pogson, C.I. (1975) FEBS Lett. 53, 29–32
16 Patel, T.B., Waymach, P.P. and Olson, M.S. (1980) Arch. Biochem. Biophys. 201, 629–635
17 Paradies, G. (1984) Biochim. Biophys. Acta 766, 446–450
18 Halestrap A.P. (1976) Biochem. J. 156, 181–183
19 Tyler, D.D. and Gouse, J. (1976) Methods Enzymol. 10, 75–77
20 Sherman, M.S. and Halestrap, A.P. (1984) Biochem. J. 223, 673–676