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INTERACTION OF α-CYANO[^{14}C]CINNAMATE WITH THE MITOCHONDRIAL PYRUVATE TRANSLOCATOR

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The binding of α-cyanocinnamate to rat-heart mitochondrial membrane was investigated using α-cyano[^{14}C]cinnamate. The binding was correlated to the inhibition of pyruvate transport. The results obtained demonstrate that both these functions reach saturation at the same titre of the inhibitor. Quantitative parameters of α-cyano[^{14}C]cinnamate binding have been determined. The binding can be prevented by pyruvate and other substrates of the carrier but not by acetate. Pyruvate decreases the affinity of α-cyanocinnamate binding, leaving the maximum number of binding unchanged. It is concluded that rat-heart mitochondria contain a specific site at which α-cyanocinnamate binds which is directly involved in the inhibition of pyruvate transport.

Introduction

α-Cyanocinnamate is a well-known and powerful inhibitor of pyruvate transport across the mitochondrial membrane [1–8]. It has been shown that this compound inhibits pyruvate transport by binding reversibly to SH groups of the carrier protein which are essential for the transport activity [9].

Although by indirect inhibitory studies it has been shown that mitochondria bind α-cyanocinnamate [10], no affinity measurements have so far been reported. Further insight into the molecular mechanism of pyruvate translocation in mitochondria can be obtained by binding studies using radiolabelled α-cyanocinnamate. The use of a radioactive ligand may be particularly useful for the study of the relationship between the inhibitory effect and the interaction of the ligand with the membrane. At the same time it may provide a means for the purification of its binding site.

This paper reports data on the binding properties of α-cyano[^{14}C]cinnamate with the mitochondrial membrane. The binding parameters of α-cyano[^{14}C]cinnamate to rat-heart mitochondria were determined and related to its inhibitory activity.

The results obtained demonstrate that rat-heart mitochondria contain a specific site at which the inhibitor binds which is directly involved in the inhibition of pyruvate transport.

Materials and Methods

Rat-heart mitochondria were prepared according to Ref. 11. 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/O ratios observed with pyruvate plus malate as substrate ranged from 2.5 to 2.9.

α-Cyanocarboxyl[^{14}C]cinnamic acid was syn-

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thesized in the Radiochemical Centre, Amersham. Its specific activity was 19.2 mCi/mol and its purity was 98%.

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

The standard medium used in the measurements of respiratory activity and in 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).

Measurement of binding

The binding of α-cyano[^{14}C]cinnamate to mitochondria was assayed as follows. First the mitochondria (1–2 mg mitochondrial protein/ml) were incubated at room temperature in the standard medium described above. The total volume was 1 ml. After 3 min of preincubation labelled α-cyanocinnamate was added, and 3 min later the reaction was terminated by rapid centrifugation of the mitochondria suspension in the cold at 15000 × *g*. The supernatant was then decanted and the pellet 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-cyano[^{14}C]cinnamate in the extramitochondrial space. The latter was determined in separate samples by the distribution of [^{14}C]sucrose. Mitochondrial proteins were determined by usual biuret method.

Results

α-Cyanocinnamate is a potent and specific inhibitor of the mitochondrial pyruvate carrier. Concentrations of the inhibitor as low as 0.1–1 μM have been shown to be effective, thus indicating an affinity constant within the range 10^6–10^7 M^{-1}.

α-Cyano[^{14}C]cinnamate binding curve obtained with rat-heart mitochondria under standard conditions (see Materials and Methods) is shown in Fig.

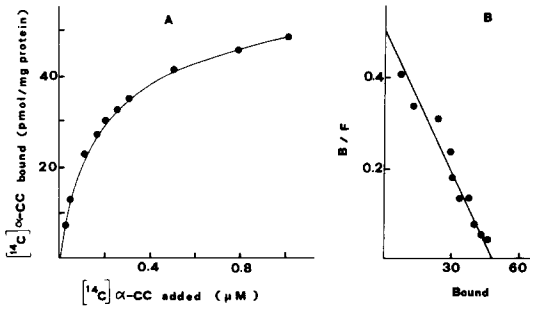

Fig. 1. Titration of α-cyano[^{14}C]cinnamate binding to rat-heart mitochondria. (A) Mitochondria (1.2 mg protein/ml) were preincubated in the standard medium as described in Materials and Methods. Final volume, 1 ml; final pH, 7.0' T, 25 °C. After 3 min increasing concentrations of labelled α-cyanocinnamate were added and 3 min later mitochondria were separated from the medium by rapid centrifugation. The binding of α-cyano[^{14}C]cinnamate ([^{14}C]α-CC) was determined as described in Materials and Methods. (B) Scatchard plot of the binding of α-cyano[^{14}C]cinnamate. Data from Fig. 1A. The number of specific binding sites (B_{max}) = 45 ± 7 pmol per mg protein; dissociation constant (K_d) = 0.095 ± 0.012 μM. The values are the means ± S.E. for five experiments

1. In the range of concentrations tested the binding of α-cyano[^{14}C]cinnamate was saturable. From these data Scatchard plots [12] were constructed (Fig. 1B), which show the presence of a specific binding site for the inhibitor. Maximal specific binding (i.e., the concentration of α-cyano[^{14}C]cinnamate binding sites), determined from a number of experimental curves similar to Fig. 1, is between 40–50 pmol/mg protein at 25 °C and at pH 7.0, while the apparent dissociation constant (K_d) is around 10^{-7} M.

In order to demonstrate that this binding site is the one at which the inhibitor is attached when pyruvate transport is inhibited, it is important first to establish that the binding parallels the inhibition of the transport. The pyruvate transport has been determined by following the rate of pyruvate-dependent oxygen uptake in the presence of ADP. Under this condition it has been shown that pyruvate translocator is rate limiting for respiration [10]. In addition it has been clearly demonstrated that the α-cyanocinnamate inhibition of pyruvate oxidation does not involve an interaction between the pyruvate dehydrogenase of intact mitochondria and α-cyanocinnamate [1,13,14]. The relationship between the binding of α-

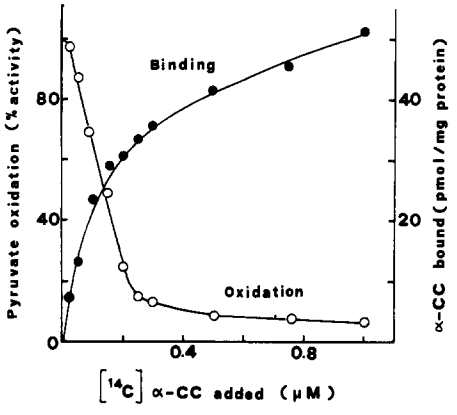

Fig. 2. α-Cyano[^{14}C]cinnamate ([^{14}C]α-CC) binding to rat-heart mitochondria and correlation to the inhibition of pyruvate oxidation. The pyruvate-dependent oxygen uptake was measured with a Clark-type electrode. Mitochondria (1.1 mg protein/ml) were preincubated in the standard medium, described in Materials and Methods, in the presence of 2 mM ADP. Final pH, 7.0; T, 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-cyano[^{14}C]cinnamate was added in the incubation mixture 3 min prior the addition of pyruvate. The rate of pyruvate oxidation, in the absence of the inhibitor, amounted to 200 ± 20 natoms O_2 per min/mg protein. This value was taken as 100% of respiratory activity. The binding of α-cyano[^{14}C]cinnamate was determined as described in Materials and Methods. ●, α-cyano[^{14}C]cinnamate binding; ○, percentage pyruvate oxidation.

cyano[^{14}C]cinnamate to the specific site and the inhibition of pyruvate oxidation is shown in Fig. 2. A close correlation between the binding of α-cyanocinnamate and the inhibition of pyruvate oxidation can be observed, both functions reaching saturation at the same titre of the inhibitor. Only at very low concentrations of added α-cyano[^{14}C]cinnamate is the binding extent more prominent than the inhibitory effect. When less than 10 pmol/mg protein of the inhibitor was bound pyruvate oxidation was only slightly inhibited, the inhibition becoming practically complete on binding about 40 pmol of inhibitor per mg of protein.

The inhibition of pyruvate oxidation by α-cyanocinnamate was analized by Dixon plots (1 per rate of oxygen uptake against inhibitor concentrations). The K_i value obtained (data taken from five experiments similar to the one reported in Fig. 2) was 0.080 ± 0.012 μM, about the same as the K_d value of α-cyanocinnamate binding.

TABLE I

THE EFFECT OF PYRUVATE AND OTHER MONOCARBOXYLIC ACIDS ON THE BINDING OF α-CYANO [^{14}C]CINNAMATE IN RAT-HEART MITOCHONDRIA

The binding of α-cyano[^{14}C]cinnamate was measured as described in the legend to Fig. 1 (A). Mitochondria (1.2 mg protein/ml) were preincubated in the standard medium described under Materials and Methods. After 3 min of preincubation α-cyano[^{14}C]cinnamate (0.2 μM) was added and 3 min later mitochondria were separated from the medium by rapid centrifugation. Pyruvate and the other monocarboxylic acids were added in the preincubation phase at the concentration of 2 mM. The results are expressed as means ± S.E. of four separate observations. For other experimental details, see the legend to Fig. 1 and in Materials and Methods.

Addition	Bound α-cyano[^{14}C]-cinnamate (pmol per mg protein)	Percentage of inhibition
None	28.7 ± 3.2	
Pyruvate	12.7 ± 1.4	55.5
2-Oxobutyrate	15 ± 1.8	47.4
Acetoacetate	17.9 ± 2.2	37.6
Monochloroacetate	14.8 ± 1.8	48.5
Phenylpyruvate	11.3 ± 1.5	61.0
Acetate	28.6 ± 2.9	1.0

The binding data of Fig. 2 were also analyzed by Scatchard plots, the results (not shown) giving quantitative parameters of binding practically identical to those reported in Fig. 1.

A further indication that the binding of α-cyanocinnamate to its specific site on mitochondrial membrane is involved in the inhibition of pyruvate transport, can be deduced from the results reported in the experiment of Table I. These show the effect of pyruvate and other monocarboxylic acids tested on their ability to affect the specific binding of α-cyano[^{14}C]cinnamate. In this experiment the inhibitor was used to a final concentration of 0.2 μM corresponding to the high-affinity portion of the binding curve. As shown in Table I, pyruvate and other substrates of the carrier such as acetoacetate, 2-oxobutyrate, monochloroacetate and phenylpyruvate were able to considerably inhibit the binding, whilst acetate, which is not transported by the pyruvate carrier [5,15], was uneffective in this respect. Interesting

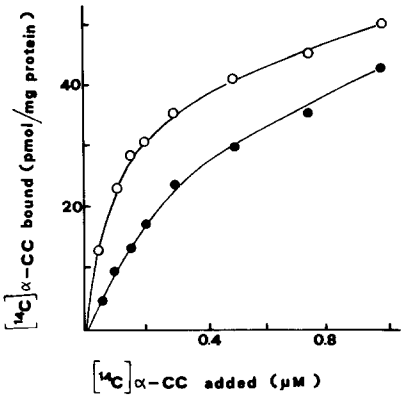

Fig. 3. The effect of pyruvate on the binding of increasing concentrations of α-cyano[^{14}C]cinnamate ([^{14}C]α-CC) to rat-heart mitochondria. The binding of α-cyano[^{14}C]cinnamate was determined as described in the legend to Fig. 1A and in Materials and Methods. Mitochondria (1 mg protein/ml) were preincubated in the standard medium described in Materials and Methods. After 3 min preincubation, increasing concentrations of labelled α-cyanocinnamate were added and 3 min later mitochondria were separated from the medium by rapid centrifugation. Pyruvate (2 mM) was added in the preincubation phase. ○, control; ●, pyruvate in the preincubation phase.

enough, phenylpyruvate was the most effective, among the substrates tested, in preventing the binding of α-cyano[^{14}C]cinnamate to its specific site. This could be due to the hydrophobic nature of this compound which makes it more accessible to the microenvironment in which the inhibitor binding site is located. In this respect it should be recalled that the inhibition of pyruvate transport by α-cyanocinnamate derivatives has been shown to be critically dependent on the hydrophobic moiety of the molecule [10].

In order to explore better the interaction of pyruvate with the α-cyanocinnamate binding site, the effect of pyruvate on the binding of increasing concentrations of the labelled inhibitor was examined. The results of such experiment are reported in Fig. 3. The inhibition of α-cyanocinnamate binding by pyruvate is much higher at the lower than at the higher concentrations of the inhibitor. From the data of Fig. 3, a K_i value for inhibition by pyruvate of α-cyanocinnamate binding of about 0.7 mM was evaluated, consistent with the value for the K_m of pyruvate transport [5,16], but much higher than the K_i value for α-cyanocinnamate inhibition of pyruvate oxidation above reported. The data of Fig. 3 were analized by Scatchard plots and the results are reported in Fig. 4. It can be observed that whilst the maximum number of α-cyanocinnamate binding remains practically unchanged, the affinity is strongly decreased, the K_d values being 0.090 ± 0.014 μM and 0.320 ± 0.040 μM in the absence and in the presence of pyruvate, respectively. This result demonstrates a competitive nature of pyruvate interaction with α-cyanocinnamate binding to mitochondria.

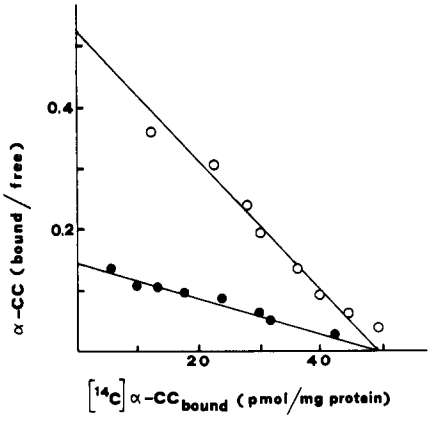

Fig. 4. Scatchard plots of α-cyano[^{14}C]cinnamate ([^{14}C]α-CC) binding to rat-heart mitochondria and the effect of inhibition by pyruvate. Data form Fig. 3.

Discussion

This paper presents the first direct study of α-cyanocinnamate binding to mitochondria. The results presented demonstrate that rat-heart mitochondria contain a specific site at which α-cyanocinnamate binds, which is directly involved in the inhibition of pyruvate transport. This conclusion is based on the following experimental observations reported in the present investigation.

(i) The binding of α-cyanocinnamate reaches saturation at the same level as that at which the pyruvate transport, measured as pyruvate supported oxygen uptake, is fully inhibited. This result demonstrates that most, if not all binding sites for α-cyanocinnamate are linked to pyruvate transport. Therefore, the binding of α-cyanocinnamate can be considered to give the most direct data on the number of carrier sites in mitochondria.

(ii) The dissociation constant (K_d) for the binding of α-cyanocinnamate is around 10^{-7} M, a value which is in the same order of magnitude as the concentration of the inhibitor required for half-maximal inhibition of pyruvate transport [6,10] and practically identical to the K_i value for the inhibition of pyruvate oxidation (present work).

(iii) The binding of α-cyanocinnamate is specifically and strongly inhibited by pyruvate and other substrates of the carrier but not by acetate. α-Cyanocinnamate is a weak acid, thus, it would distribute across the mitochondrial membrane according to the transmembrane ΔpH. The inhibition of α-cyanocinnamate binding by pyruvate and other substrates of the carrier might therefore be due to a decrease of ΔpH induced by the uptake of these substrates. However, the lack of inhibition by acetate, whose distribution across the membrane follows the pH gradient as well Ref. 17, minimizes this possibility, whilst suggesting a direct interaction of pyruvate with the α-cyanocinnamate binding site. This, in addition to the competitive nature of the effect of inhibition of α-cyanocinnamate binding by pyruvate (see Fig. 4), support the view that both substrates and inhibitor bind very near to or at the same site on the carrier molecule.

It has been clearly shown that the molecular mechanism of α-cyanocinnamate inhibition of pyruvate transport in mitochondria involves a reversible binding to SH groups which are essential for the activity of the transporting system [9]. Therefore, unless there is an alternative or additive mode of binding of the inhibitor to the carrier protein, the quantitative estimation of the α-cyanocinnamate binding sites can furnish the total number of SH groups involved in the pyruvate translocation. This number amounts to 45 pmoles/mg protein, a value which is much lower than the one reported by other authors [10], who estimated the number of carrier molecules by indirect inhibitory measurements, but very similar to the number of SH groups reported for the phosphate carrier in rat-liver mitochondria [18–21].

The nonlinear relation between the inhibition of pyruvate oxidation and saturation of the binding site observed at lower concentrations of the inhibitor (see Fig. 2), suggests that some of these SH groups might not be essential for the inhibitory activity, or alternatively that pyruvate transport has some spare capacity for pyruvate oxidation under the experimental conditions used.

Further studies on the characterization of α-cyanocinnamate binding to mitochondria and submitochondrial particles will be particularly useful in elucidating the role of the thyol groups in the functioning of the pyruvate carrier as well as in getting more insight into its molecular mechanism.

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