ELSEVIER

Biochimica et Biophysica Acta 1234 (1995) 149–154

BBA Biochimica et Biophysica Acta

Photoaffinity labeling of the mitochondrial oxoglutarate carrier by azido-phthalonate

Italo Stipani [a,*], Dorotea Natuzzi [a], Lucia Daddabbo [a], Alberto Ritieni [b], Giacomino Randazzo [b], Ferdinando Palmieri [a]

[a] *Department of Pharmaco-Biology, Laboratory of Biochemistry and Molecular Biology, University of Bari, CNR Unit for the Study of Mitochondria and Bioenergetics, Via E. Orabona 4, 70125 Bari, Italy*

[b] *Department of Food Science, University of Napoli Federico II, Napoli, Italy*

Received 1 August 1994; accepted 24 November 1994

Abstract

The effect of azido-phthalonate, a photoreactive analogue of oxoglutarate, on the transport of oxoglutarate was investigated in proteoliposomes reconstituted with the purified oxoglutarate carrier. In the dark, azido-phthalonate inhibits the reconstituted oxoglutarate/oxoglutarate exchange in a competitive manner with a K_i of 0.38 mM. Upon photoirradiation, the inhibition of the oxoglutarate exchange by azido-phthalonate is not removed by passing the proteoliposomes through a Sephadex column. The light-induced inhibition of the oxoglutarate/oxoglutarate exchange activity by azido-phthalonate is time- and concentration-dependent. The kinetic analysis of transport inhibition by azido-phthalonate reveals that one molecule of this substrate analogue bound to the functional carrier molecule is responsible for complete inhibition of the carrier function. Azido-[^{3}H]phthalonate binds to the oxoglutarate carrier covalently. Incubation of the proteoliposomes with oxoglutarate during photoirradiation in the presence of azido-phthalonate protects the carrier against inactivation and decreases the amount of radioactivity which is found to be associated with the carrier protein. It is concluded that azido-phthalonate can be used for photoaffinity labeling of the mitochondrial oxoglutarate carrier at the substrate-binding site.

Keywords: Oxoglutarate carrier; Azidophthalonate; Photoaffinity labeling; Transport; Mitochondrion; (Bovine heart)

1. Introduction

The inner mitochondrial membrane contains a specific carrier system that catalyzes a counterexchange between oxoglutarate and malate (for review, see Refs. [1] and [2]). The oxoglutarate carrier plays an important role in several metabolic processes like gluconeogenesis from lactate and the malate-aspartate shuttle. In our laboratory the mitochondrial oxoglutarate carrier was isolated from heart and liver and functionally reconstituted into liposomes [3,4]. Kinetically, the carrier functions according to a simultaneous (sequential) mechanism [5,6]. In 1990, the amino acid sequence of the oxoglutarate carrier was determined by cDNA sequencing [7]. More recently, the oxoglutarate carrier was overexpressed in *Escherichia coli* and refolded in active form [8]. Furthermore, a great deal of information on the transmembrane arrangement of this carrier in the mitochondrial membrane has been obtained [9]. Although there is such a large amount of information regarding the functional and structural properties of the oxoglutarate carrier no studies have yet been performed towards the identification of the substrate-binding sites.

The substrate-analogue phthalonate is a strong and competitive inhibitor of the oxoglutarate carrier [10]. With the aim of labeling the substrate-binding site of the oxoglutarate carrier, we synthesized azido-phthalonate and investigated its effects on the purified oxoglutarate carrier reconstituted into liposomes. Our results show that azido-phthalonate binds to the oxoglutarate carrier covalently upon photoirradiation and one molecule of azido-phthalonate modifies one molecule of the functional carrier unit.

Abbreviations: SDS, sodium dodecylsulfate; Pipes, 1,4-piperazine-diethanesulfonic acid.

* Corresponding author. Fax: +39 80 5442770.

SSDI 0005-2736(94)00291-6

2. Materials and methods

2.1. Materials

Hydroxyapatite (Bio-Gel HTP), AG1-X4 and AG1-X8 were purchased from Bio-Rad; Celite 535 from Roth, AMPLIFY and 2-keto[5-^{14}C]glutaric acid from Radiochemical Centre (Amersham, UK); Pipes and Triton X-114 from Sigma; pyridoxal 5′-phosphate from Serva; 5-aminotetralin and Amberlite XAD-2 from Fluka; egg yolk phospholipids from Merck; Sephadex G-75 from Pharmacia. All other reagents and solvents were of the highest purity commercially available.

2.2. Isolation and reconstitution of the oxoglutarate carrier

The oxoglutarate carrier from bovine heart mitochondria was purified in Triton X-114 by the procedure introduced by Bisaccia et al. [3]. Reconstitution of the carrier into liposomes was performed by removing the detergent with a hydrophobic ion-exchange column [6,11]. The composition of the starting mixture used for reconstitution was: 200 μl of the purified protein (hydroxyapatite/celite eluate, about 2 μg), 100 μl of 10% egg yolk phospholipids in the form of sonicated liposomes prepared as described in Ref. [3], 10 mM oxoglutarate and 10 mM Pipes, pH 7, in a final volume of 700 μl. After vortexing, this mixture was passed 15 times through the same Amberlite column (0.5 × 2.5 cm), preequilibrated with a buffer containing 10 mM Pipes, pH 7. All the operations were performed at 4° C, except the passages through Amberlite that were performed at room temperature. The external substrate was removed by passing 600 μl proteoliposomes through a Sephadex G-75 column (0.7 × 15 cm) preequilibrated with 50 mM NaCl and 10 mM Pipes, pH 7.

2.3. Transport measurements

The first 600 μl of the turbid eluate from the Sephadex column were collected, transferred to reaction vessels (150 μl each), incubated at 25° C for 4 min and then used for transport measurements by the inhibitor stop method [12]. Transport was initiated by adding 0.1 mM [^{14}C]oxoglutarate and stopped, 1 min later, by adding 10 mM pyridoxal 5′-phosphate. In control samples, the inhibitor was added together with the labeled substrate. The assay temperature was 25° C. Finally, the external radioactivity was removed by an anion-exchange column (AG1-X8, 100–200 mesh, acetate form, 0.5 × 4 cm). The liposomes eluted with 1 ml of 50 mM NaCl were collected in a 4 ml scintillation mixture, vortexed and counted. The transport activity was calculated by subtracting the control from the experimental values. In all the experiments the oxoglutarate/oxoglutarate exchange activity in the samples without inhibitor varied from 3200 to 4350 μmol/min per g protein.

2.4. Synthesis and purification of azido-phthalonic acid

The precursor of azido-phthalonic acid, 5-azido-tetralin (**2** of Fig. 1), was synthesized from amino-tetralin (**1** of Fig. 1) according to Heilbrun et al. [13] with a yield of 75%. The purity of 5-azido-tetralin was verified by TLC eluted with ethyl acetate/n-hexane (30:70, v/v; R_F = 0.85) and the characteristic absorption IR band at 2933 cm^{-1} confirmed the presence of an azido group. For the synthesis of azido-phthalonic acid (**3** of Fig. 1), 17 ml of 0.89 M $KMnO_4$ were added dropwise, at room temperature, in 1.5 h to a solution of 500 mg of 5-azido-tetralin suspended in 5 ml of 0.5 M NaOH. The mixture was stirred for 30 min and the temperature reaction was then increased and maintained constant at 75° C for 1 h. The reaction was monitored by SiO_2 TLC eluted by n-butanol/water/acetic acid (60:25:15 v/v). The MnO_2 was then filtered and the solution was acidified at pH 1 with H_2SO_4 and extracted with ethyl acetate (three times 10 ml). The organic phases were pooled, dried on Na_2SO_4 and, after removal of the solvent at reduced pressure, 292 mg of the crude product was obtained with R_F = 0.4 in the above solvent system. The preliminary step of purification was performed by an AG1-X4 column (200–400 mesh, 1.5 × 15 cm), eluted with formic acid 15 M [14]. Azido-phthalonic acid (180 mg, yield 27%) was obtained as white amorphous solid by preparative TLC eluted with n-butanol/water/acetic acid (60:25:15, v/v). The purity of the product was checked by TLC on silica gel F_{254} (Merck). The molecular structure was confirmed by ^{13}C-NMR, ^{1}H-NMR, IR and MS.

2.5. Synthesis of azido-[^{3}H]phthalonic acid

102 mg of azido-phthalonic acid were dissolved in 1 ml of dimethylformamide using 102 mg of 5% Rh/Al_2O_3 as catalyst. 25 Ci of tritiated water were added to this solution. The reaction was stirred for 12 h at room temperature. Labiles were removed with methanol. The specific activity was 1.87 GBq/mmol. Azido-[^{3}H]phthalonic acid was obtained from the Tritium Labeling Service (Du Pont).

2.6. Covalent modification of the oxoglutarate carrier

Proteoliposomes (2 ml) were photoirradiated in the presence of azido-phthalonate or azido-[^{3}H]phthalonate (specific radioactivity 50.7 mCi/mmol) at 0° C for 20 min

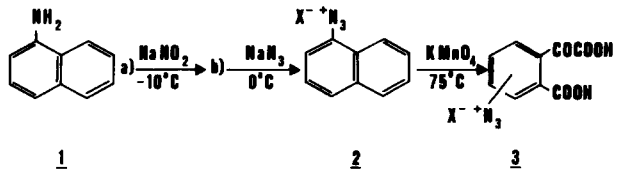

Fig. 1. Synthesis of azido-phthalonic acid.

(unless otherwise indicated). Photoirradiation was carried out with a 250 W Osram Halogen lamp at 0° C under shaking and in quartz tubes. The modification reaction was stopped by switching off the light and rapidly passing the liposomes through a Sephadex G-75 column preequilibrated with 50 mM NaCl and 10 mM Pipes, pH 7.

2.7. Other methods

In order to extract the oxoglutarate carrier from the proteoliposomes, the photoirradiated proteoliposomes (7.5 ml) were precipitated with cold acetone and the lipids were carefully removed as described in Ref. [9]. Polyacrylamide slab-gel electrophoresis was performed in the presence of 0.1% SDS according to Laemmli [15]. The stacking gel contained 5% acrylamide and the separation gel contained 17.5% acrylamide and an acrylamide/bisacrylamide ratio of 150. Staining was performed with Coomassie brilliant blue. For fluorographic detection of radioactivity, the stained gels were treated with AMPLIFY for 30 min and dried on filter paper in vacuo (70° C, 90 min). The fluorographic exposure of a non pre-flashed X-ray film (Kodak X-OMAT) took 4–6 weeks at −80° C. Protein was determined by the Lowry method modified for the presence of Triton [16].

^{1}H-NMR and ^{13}C-NMR spectra were recorded at 30° C on a Bruker AM400. ^{13}C-NMR spectra were performed at 100.5 MHz using the products dissolved in CD_3OD. The IR spectra were performed by Perkin Elmer 399; the low resolution electronic mass spectrometry data were obtained using a TRIO 2000 Fisons at 70 eV, 400 μA and a source temperature of 200° C.

3. Results

3.1. Synthesis of azido-phthalonic acid

The oxidation reaction of 5-azido-tetralin had to be performed at basic pH so as to lower the oxidation power of $KMnO_4$. Moreover, the temperature reaction had to be held at 75° C; above this temperature the yield of azido-phthalonic acid decreased while azido-phthalic acid began to form. The identity of azido-phthalonic acid was confirmed by means of spectroscopic methods. A characteristic absorption band at 3000 cm^{-1} of the azido group was present in IR (Nujol) spectrum. Moreover, ^{13}C-NMR (D20) spectrum showed the following chemical shifts: δ: 190.97 (Ph-C=O), 174.41 (Ph-COOH) and 165.80 (Ph-CO-COOH); the ^{1}H-NMR spectrum was also in agreement with the proposed structure. The MS spectrum recorded showed the ion molecular peak at m/z 235 (M^+), the molecular peak with loss of CO_2 at m/z 191 and an ion peak at m/z 177 produced by loss of CO_2 and nitrogen.

3.2. Inhibition of the reconstituted oxoglutarate carrier by azido-phthalonate without illumination

The effect of the newly synthesized compound azido-phthalonate on the transport of oxoglutarate in proteoliposomes without irradiation is shown in Fig. 2 as a Lineweaver-Burk plot. Azido-phthalonate inhibited the reconstituted oxoglutarate/oxoglutarate exchange activity in proteoliposomes in a competitive manner with respect to the substrate. From this and other experiments a K_i of 0.38 ± 0.03 mM (in five determinations) was estimated.

3.3. Inhibition of the reconstituted oxoglutarate carrier by azido-phthalonate upon illumination

It is known that azido compounds photolyse on exposure to ultraviolet light to form a highly reactive nitrene. This nitrene is able to react covalently with its environment [17]. In the following experiments the effect of azido-phthalonate on the reconstituted oxoglutarate carrier activity was investigated upon photoirradiation. Fig. 3A illustrates the dependence of the light-induced azido-phthalonate inhibition of the reconstituted oxoglutarate/oxoglutarate exchange on the concentration of the inhibitor. In this experiment proteoliposomes were illuminated in the presence of azido-phthalonate. After removal of the unbound azido-phthalonate by gel filtration, the reconstituted oxoglutarate/oxoglutarate exchange activity was measured. The results show that the inhibition of the oxoglutarate carrier activity increased with increasing concentrations of azido-phthalonate with an IC_{50} of 0.4 mM. As a control, Fig. 3A shows that the activity of the

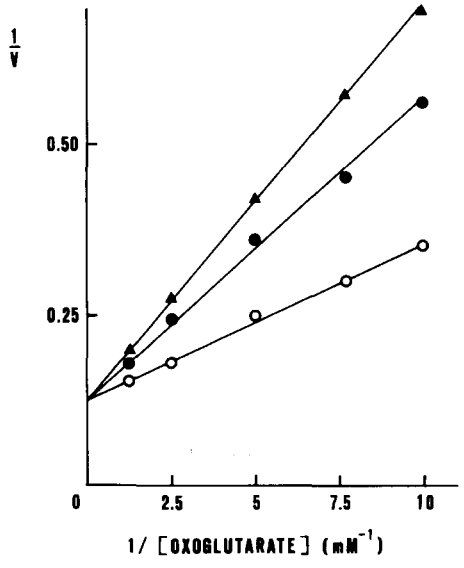

Fig. 2. Competitive inhibition of oxoglutarate transport by azido-phthalonate in the dark. The proteoliposomes were incubated for 1 min at 25° C in the dark with the indicated concentrations of [^{14}C]oxoglutarate in the absence (○) or in the presence of 0.35 mM (●) or 0.7 mM (▲) azido-phthalonate. V is expressed in μmol/min per mg protein.

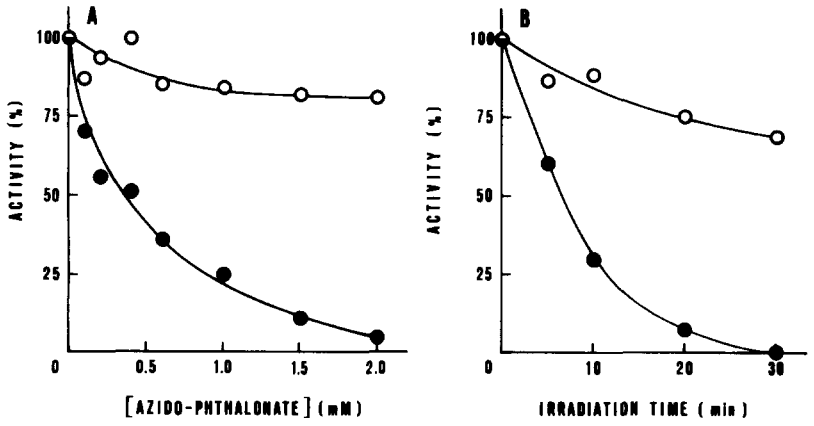

Fig. 3. (A) Dependence of the inhibition of the reconstituted oxoglutarate carrier activity on azido-phthalonate concentration in the dark and upon irradiation. The proteoliposomes were incubated for 20 min at 0° C with the indicated concentrations of azido-phthalonate with (●) or without (○) irradiation. After a second gel filtration to remove the unbound inhibitor, the oxoglutarate/oxoglutarate exchange was measured. (B) Dependence of the inhibition of the reconstituted oxoglutarate carrier activity by azido-phthalonate on the photoirradiation time. The proteoliposomes were irradiated for the times indicated at 0° C in the presence (●) or absence (○) of 2 mM azido-phthalonate. After a second gel filtration to remove the unbound inhibitor, the oxoglutarate/oxoglutarate exchange was measured.

oxoglutarate carrier was not influenced by azido-phthalonate without photoirradiation, in agreement with the finding that the inhibition by azido-phthalonate in the dark is reversible (Fig. 2). The effect of the photoirradiation time on the oxoglutarate transport activity in the presence and absence of 2 mM azido-phthalonate is shown in Fig. 3B. It can be seen that photoirradiation of the proteoliposomes alone, i.e., in the absence of azido-phthalonate, caused a slight inhibition of oxoglutarate transport, which reaches a value of 30% after 30 min of illumination. For this reason in all the other experiments the proteoliposomes were illuminated for 20 min and the inactivation caused by this treatment was always subtracted from the inhibition values obtained in the presence of azido-phthalonate. Fig. 4A shows the semilogarithmic plots of the remaining activity with azido-phthalonate versus time. The data fitted straight-lines, the slope of which allowed one to calculate apparent first-order rate constants of the inactivation reaction (with respect to the protein) for different inhibitor concentrations [18]. From the slope of double-logarithmic plot (Fig. 4B) the reaction order with respect to azido-phthalonate could be determined to be $n = 0.71 \pm 0.056$. This value suggests that the reaction of one molecule of azido-phthalonate per functional carrier molecule lead to complete inhibition of the carrier function.

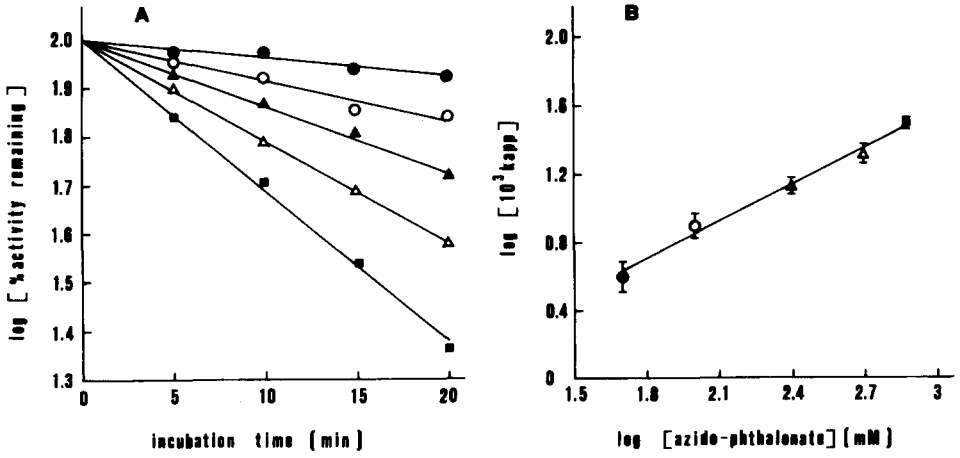

Fig. 4. Kinetics of inactivation of the reconstituted oxoglutarate carrier by azido-phthalonate. The proteoliposomes were illuminated at 0° C in the presence of different concentrations of azido-phthalonate (●, 0.05 mM; ○, 0.1 mM; ▲, 0.25 mM; △, 0.5 mM; ■, 0.75 mM) for the indicated times. The modification reaction was stopped by switching off the light and rapidly passing the proteoliposomes through a Sephadex G-75 column. Then the residual activity was measured, which is given as percentage of controls (A). In (A) the means of four experiments are reported. In (B) the double-logarithmic secondary plot of the means ± S.D. of the apparent inactivation constant K_{app} (2.3 × slope of the straight lines of A) versus the applied azido-phthalonate concentration is shown.

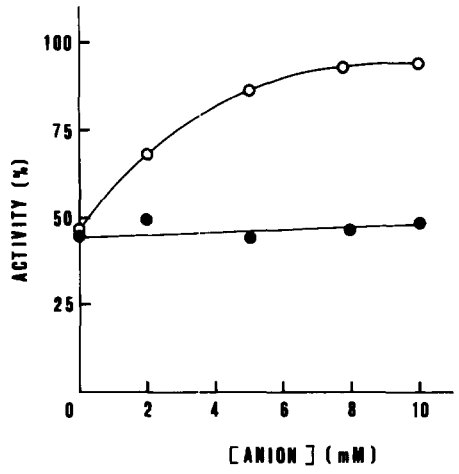

Fig. 5. Substrate protection against inactivation by azido-phthalonate. The proteoliposomes were irradiated for 20 min at 0° C in the presence of 0.4 mM azido-phthalonate and the indicated concentrations of oxoglutarate (○) or glutarate (●). After a second gel filtration to remove the unbound inhibitor the residual activity was measured.

On the basis of the competition experiments it can be assumed that azido-phthalonate binds to the oxoglutarate carrier at the substrate binding-site. Therefore, it was important to test whether oxoglutarate correspondingly protects the oxoglutarate carrier from inactivation by azido-phthalonate. Fig. 5 shows that the light-induced inhibition of the oxoglutarate transport activity by 0.4 mM azido-phthalonate was progressively reversed by the presence of increasing concentrations of oxoglutarate during photoirradiation. The specificity of the protection of the oxoglutarate carrier against azido-phthalonate inactivation was investigated by testing the effect of several anions. As shown in Fig. 5, glutarate up to 10 mM had no protective effect. Similarly, ADP, phosphate and oxoadipate had virtually no effect on the light-induced inhibition of the oxoglutarate carrier by azido-phthalonate (not shown).

3.4. Labeling of the oxoglutarate carrier by azido-[^{3}H]phthalonate

In the experiment illustrated in Fig. 6 the oxoglutarate carrier extracted from the proteoliposomes photoirradiated in the presence of 1 mM azido-[^{3}H]phthalonate was subjected to SDS-polyacrylamide gel electrophoresis and fluorography. Lane 2 of Fig. 6B shows that azido-[^{3}H]phthalonate bound to the purified oxoglutarate carrier which corresponds to a band of an apparent molecular mass of 31.5 kDa (lanes 1–4 of Fig. 6A and Ref. [3]). As shown by lane 3 of Fig. 6B the labeling of the oxoglutarate carrier by azido-[^{3}H]phthalonate was drastically reduced when oxoglutarate was present during the incubation of the proteoliposomes with the inhibitor in the light. In contrast, the labeling of the carrier protein was not significantly influenced (lane 4 of Fig. 6B) when the proteoliposomes were incubated with azido-[^{3}H]phthalonate in the light in the presence of glutarate. This agrees with the substrate protection of the carrier against inactivation by azido-phthalonate (Fig. 5).

4. Discussion

The results presented in this paper show that azido-phthalonate without illumination inhibits the oxoglutarate carrier competitively. Its inhibition constant ($K_i = 0.38$ mM) is not significantly different from that exhibited, under identical experimental conditions, by phthalonate ($K_i = 0.35$ mM), the well known competitive and impermeable inhibitor of the oxoglutarate carrier [10]. As compared to the affinity of the two substrates for the oxoglutarate carrier, the affinity of the new inhibitor azido-phthalonate is slightly lower than that for oxoglutarate and definitively higher than that for malate [6]. When the

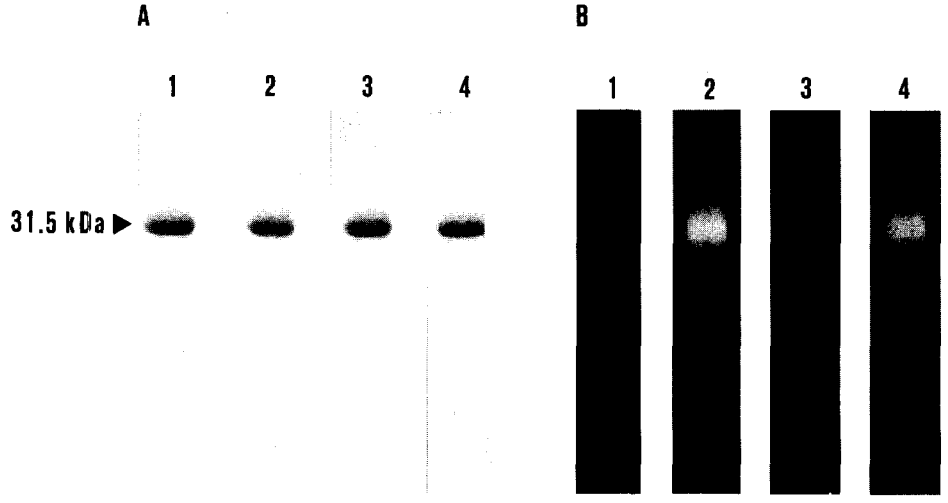

Fig. 6. Labeling of the oxoglutarate carrier in proteoliposomes by azido-[^{3}H]phthalonate. The proteoliposomes (7.5 ml) were incubated for 20 min at 0° C with 1 mM azido-[^{3}H]phthalonate in the light (lanes 2–4) or in the dark (lane 1), in the absence (lanes 1,2) or in the presence of 5 mM oxoglutarate (lane 3) or 5 mM glutarate (lane 4). After removal of the unbound azido-phthalonate the oxoglutarate carrier protein was delipidated and subjected to SDS-gel electrophoresis and fluorography. (A) SDS-polyacrylamide gel electrophoresis; (B) fluorography.

proteoliposomes are incubated with azido-phthalonate and photoirradiated, the oxoglutarate carrier becomes irreversibly modified, as shown by the irreversible inhibition of the carrier activity (Fig. 3A) and by the radioactivity associated with the carrier (Fig. 6). Most importantly, several experimental observations indicate that azido-phthalonate binds to the oxoglutarate carrier at the substrate-binding site. The strongest argument in favour of this conclusion is the substrate protection of the oxoglutarate carrier against the inactivation caused by azido-phthalonate in the light (Fig. 5) and its binding to the carrier protein (Fig. 6). The kinetic analysis of the reconstituted oxoglutarate transport inactivation suggests that one molecule of azido-phthalonate modifies one molecule of the functional carrier unit (Fig. 4). This result could be explained by assuming that the oxoglutarate carrier has two independent substrate-binding sites, one on the external and the other on the internal side of the membrane (see Ref. [6]), and the azido-phthalonate, being impermeable, binds only at the external surface.

In conclusion, we have shown that azido-phthalonate is a suitable label to characterize the oxoglutarate-binding site of the mitochondrial oxoglutarate carrier. Experiments are in progress to identify the region of the oxoglutarate carrier modified by azido-phthalonate.

Acknowledgements

This work was supported by the Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MURST).

References

[1] LaNoue, K.F. and Schoolwerth, A.C. (1979) Annu. Rev. Biochem. 48, 871–922.
[2] Kramer, R. and Palmieri, F. (1992) in Molecular Mechanisms in Bioenergetics (Ernster, L., ed.), pp. 359–384, Elsevier, Amsterdam.
[3] Bisaccia, F., Indiveri, C. and Palmieri, F. (1985) Biochim. Biophys. Acta 810, 362–369.
[4] Bisaccia, F., Indiveri, C. and Palmieri, F. (1988) Biochim. Biophys. Acta 933, 229–240.
[5] Sluse, F.E., Ranson, M. and Liebecq, C. (1972) Eur. J. Biochem. 25, 207–217.
[6] Indiveri, C., Dierks, T., Kramer, R. and Palmieri, F. (1991) Eur. J. Biochem. 198, 339–347.
[7] Runswick, M.J., Walker, J.E., Bisaccia, F., Iacobazzi, V. and Palmieri, F. (1990) Biochemistry 29, 11033–11040.
[8] Fiermonte, G., Walker, J.E. and Palmieri, F. (1993) Biochem. J. 294, 293–299.
[9] Bisaccia, F., Capobianco, L., Brandolin, G. and Palmieri, F. (1994) Biochemistry 33, 3705–3713.
[10] Meijer, A.J., Van Woerkom, G.M. and Eggelte, T.A. (1976) Biochim. Biophys. Acta 430, 53–61.
[11] Kramer, R. and Heberger, G. (1986) Biochim. Biophys. Acta 868, 289–296.
[12] Palmieri, F. and Klingenberg M. (1972) Methods Enzymol. 56, 222–232.
[13] Heilbrun, L., Brenbury, C. (1978) Dictionary of Organic compounds Vol. 2, pp. 635–637, Eyre and Spottiswoode Publishers, London.
[14] Davies, C., Hartley, G. and Lawson, G.J. (1965) J. Chromatogr. 18, 47–52.
[15] Laemmli, U.K. (1970) Nature 227, 680–685.
[16] Dulley, J.R. and Grieve, P.A. (1975) Anal. Biochem. 74, 136–141.
[17] Knowles, J.R. (1972) Acc. Chem. Res. 5, 155–160.
[18] Levy, H.M., Leber, P.D. and Ryan, E.M. (1963) J. Biol. Chem. 238, 3654–3659.