Volume 261, number 1, 39-42 FEBS 08100 February 1990

The monocarboxylate carrier from rat liver mitochondria

Purification and kinetic characterization in a reconstituted system

Ferdinando Capuano, Marco Di Paola, Angelo Azzi* and Sergio Papa

Institute of Medical Biochemistry and Chemistry, University of Bari, Bari, Italy and **Institute fur Biochemie und Molekularbiologie der Universitat Bern, Bühlstrasse 28, CH-3012 Bern, Switzerland*

Received 14 December 1989

The monocarboxylate (pyruvate) carrier was extracted from rat liver mitochondria with Triton X-100 in the presence of asolectin and partially purified by chromatography on HTP. The HTP eluate reconstituted in liposomes was shown to catalyze active $pyruvate_{in}$/$acetoacetate_{out}$ and $acetoacetate_{in}$/$pyruvate_{out}$ counter-exchange. Kinetic characterization of the reconstituted pyruvate carrier was achieved by an original spectrophotometric method consisting of determination of substrate release from proteoliposomes with a coupled enzymatic assay.

Monocarboxylate carrier; Pyruvate transport; Reconstitution; Mitochondria; (Rat liver)

1. INTRODUCTION

The inner membrane of mitochondria contains a specific transporting system, identified for the first time in rat liver in 1971 [1], which catalyzes net pyruvic acid uptake by pyruvate/H^+ co-transport (or counter-transport of OH^-) or pyruvate/pyruvate exchange [2,3]. It was shown that, besides pyruvate, this system mediates also translocation of various monocarboxylic oxoacids which include acetoacetate, etc. [4]. Thus, the system may be named monocarboxylate translocator. The properties of the monocarboxylate carrier have been extensively investigated in intact mitochondria [5-9] but, until now, little is known about its molecular structure.

Most of the mitochondrial substrate carriers have been purified, by the use of hydroxylapatite chromatography of soluble extracts of mitochondrial proteins. The number of the different proteins identifiable in the hydroxylapatite pass-through, depends on: (i) the source of mitochondria; (ii) the type and the concentration of the detergent used for solubilization of the mitochondrial protein; (iii) the phospholipid utilized during solubilization [10]. Recently, the mitochondrial monocarboxylate translocator was purified from beef heart by passing the hydroxylapatite eluate through an affinity resin with an hydroxycinnamate derivative as ligand [11].

The isolation of mitochondrial protein carriers from liver generally appears to be more elaborated as compared to heart apparently due to the different composition of the mitochondrial membrane [2]. Here, we report on the partial purification of the monocarboxylate carrier from rat liver mitochondria obtained by chromatography on hydroxyapatite and its kinetic characterization, after incorporation in liposomes, by an original spectrophotometric method which allows rapid, continuous and direct measurement of initial rates, thus avoiding difficulties that may affect usual methods based on determination of translocation of radioactive substrates at various time intervals.

2. MATERIALS AND METHODS

2.1. *Materials*

Hydroxyapatite (Bio-Gel HTP), Dowex AG 1-X8 Cl^- form (100-200 mesh) and marker proteins were purchased from Bio-Rad. Amberlite XAD-2 beads, Triton X-100, egg yolk phospholipids were obtained from Fluka. Pyruvic acid, acetoacetic acid, 2 cyan-4-hydroxycinnamate and Asolectin were from Sigma. NADH, lactate dehydrogenase (EC 1.1.1.27), 3-hydroxybutyrate dehydrogenase (C 1.1.1.30) were from Boehringer Mannheim. Mops and Folin reagent were from Merk, Sephadex G-25M from Pharmacia.

2.2. *Preparation of mitochondria*

Rat liver mitochondria were prepared according to [13].

2.3. *Purification of pyruvate carrier*

Rat liver mitochondria were solubilized in a medium containing: 3% Triton X-100, 20 mM Mops, pH 7.2, 50 mM NaCl, 1 mM EDTA, 10 mg/ml Asolectin at a protein concentration of 15 mg/ml. After 20 min at 0°C, the mixture was centrifuged at 140000 × *g* for 1 h.

Triton X-100 extract (0.6 ml) was applied on a column containing

Correspondence address: S. Papa, Institute of Medical Biochemistry and Chemistry University of Bari, Policlinico, Piazza Giulio Cesare, 70124 Bari, Italy

Abbreviations: PAGE, polyacrylamide gel electrophoresis; HTP, hydroxylapatite; LDH, lactate dehydrogenase; 3-HBDH, 3-hydroxybutyrate dehydrogenase

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

600 mg cold dry HTP and eluted at 4°C with 1.8 ml of medium containing: 3% Triton X-100, 20 mM Mops, pH 7.2, 50 mM NaCl, 1 mM EDTA, 2 mg/ml Asolectin.

2.4. *Reconstitution of the HTP eluate*

Liposomes were prepared by sonicating 90 mg egg yolk phospholipids in 2 ml of 20 mM Mops pH 7.2, 50 mM KCl, 1 mM EDTA, under nitrogen at 4°C, using the microtip of a Branson Sonifier B15 in the pulsed mode at 50% duty, until the suspension was clarified. Amberlite XAD-2 beads (500 mg) were preequilibrated with 0.1 ml liposomes for 1 h at room temperature to minimize lipid and protein adsorption during the reconstitution. The HTP heluate (0.4 ml) was mixed with 0.1 ml of liposomes, then 200 mM pyruvate (or acetoacetate) was added. The equilibrated Amberlite XAD-2 beads were added to the mixture and gently mixed at 4°C for 3 h. Then, 500 μl of proteoliposomes were removed from the beads and quickly passed through a small anion exchange column (Pasteur pipette filled with Dowex AG 1-X8, preequilibrated with 170 mM sucrose) to remove the external substrate.

2.5. *Measurement of exchange activity*

The activity was measured by direct spectrophotometric determination of substrate release from proteoliposomes with a coupled enzymatic assay.

100 μl proteoliposomes were suspended in a reaction mixture containing: 1.4 ml of 20 mM Mops, pH 7.2, 50 mM KCl, 1 mM EDTA, in a spectrophotometric cuvette, thermostatically controlled at 25°C. 10 μl of 10 mM NADH were then added followed by 5 μl of lactate dehydrogenase (~10 U) or 3-hydroxybutyrate dehydrogenase (~10 U). The exchange reaction was started by adding under rapid stirring the counteranion. Acetoacetate or pyruvate efflux from proteoliposomes was measured by following spectrophotometrically NADH oxidation, at 360–374 nm ($\Delta\varepsilon = 2.3$ mM^{-1}) with a Johnson Foundation dual wavelength spectrophotometer.

2.6. *SDS-Polyacrylamide gel electrophoresis*

Gel electrophoresis was performed according to Laemmli [14] using a 17.5% polyacrylamide separating gel as described in [15]. The protein was precipitated by adding 5 vols of cold acetone (stored at −20°C) and dissolved in 0.1 M Tris-HCl (pH 6.8), 2% SDS, 2% mercaptoethanol, 20% glycerol. Electrophoresis was carried out at 25 mA for 3 h. Polypeptides were fixed in 6% formaldehyde and stained with silver nitrate according to the Bio-Rad procedure.

2.7. *Protein determination*

Protein was determined according to Lowry [16] modified as in [17].

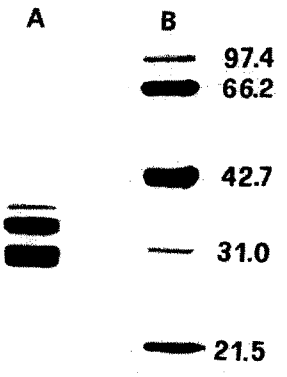

Fig.1. SDS-PAGE pattern of the HTP eluate from Triton X-100 solubilized rat liver mitochondria. About 12 μg protein of the HTP eluate (A) and 2 μg of each marker proteins (B) were applied on the slab. For details see section 2.

3. RESULTS

SDS-PAGE analysis of the hydroxyapatite eluate from solubilized rat liver mitochondria (see fig.1) shows a polypeptide pattern similar to that reported for heart mitochondria [17]. The eluate essentially contains 5 proteins in the apparent molecular mass region of 29–37 kDa. In order to verify the presence in the mitochondrial extract of an active pyruvate carrier the hydroxyapatite eluate was reconstituted, with the detergent removal method, in phospholipid vesicles. Prior to reconstitution, all fractions were passed through Sephadex G-25M in order to eliminate the excess of

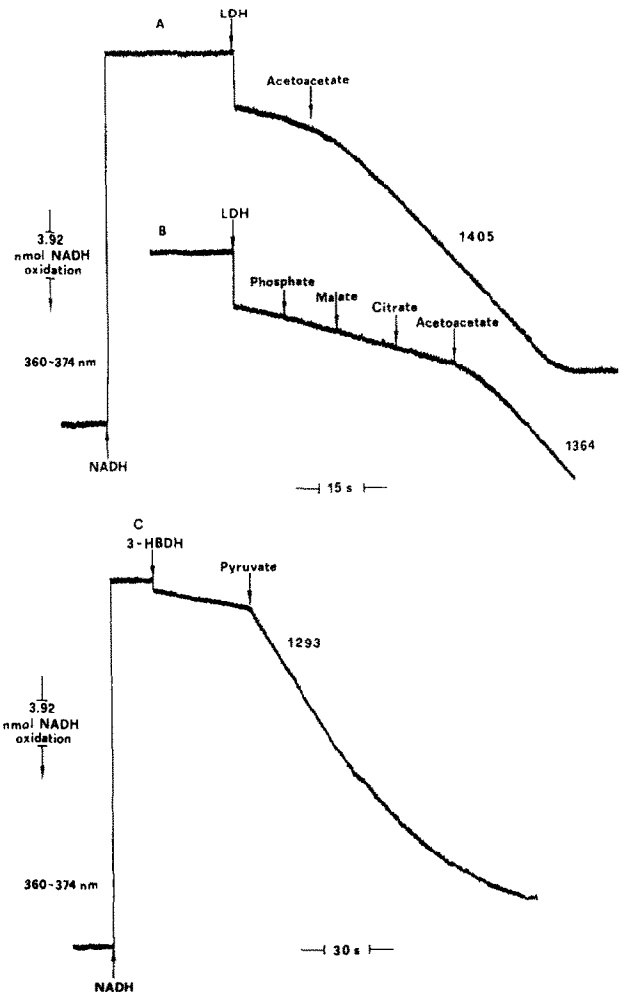

Fig.2. Pyruvate$_{in}$/acetoacetate$_{out}$ and acetoacetate$_{in}$/pyruvate$_{out}$ exchange activity of HTP eluate from Triton X-100 solubilized rat liver mitochondria reconstituted in liposomes. In (A) and (B) 100 μl of proteoliposomes (0.012 μg of protein) preloaded with 200 mM pyruvate were incubated at 25°C and pH 7.2 in the reaction medium described in section 2. (A) The reaction was started by adding 1.33 mM (final concentration) acetoacetate. The reported value of maximal velocity, expressed as nmol pyruvate exchanged per min and per mg of protein, is not corrected for the diffusion of pyruvic acid. (B) Where indicated 1.33 mM of anion was added. (C) Proteoliposomes (0.0097 μg per protein) preloaded with 200 mM acetoacetate were pulsed with 1.33 mM pyruvate. Further details of assay conditions are given in section 2.

Ca^{2+} ions present in the hydroxyapatite eluate [18]. The efflux of pyruvate from loaded proteoliposomes was followed by monitoring at 360–374 nm the oxidation of NADH by a trace of LDH added to the reaction mixture (fig.2A and B). The immediate decrease of absorbance observed upon addition of LDH, is due to the pyruvate leaked out of liposomes. The extent of this absorbance decrease increased, in fact, with the length of incubation before LDH addition. This was followed by a slow linear oxidation of NADH due to net efflux of pyruvic acid. The exchange reaction was started by the addition of acetoacetate. After a lag of 5–10 s, the oxidation of NADH reached its maximal velocity (see also table 1). Separate control showed that the delay observed was not due to the effect of mixture components on lactate dehydrogenase activity. As reported in fig.2B, anions like phosphate, malate and citrate did not promote efflux of pyruvate from proteoliposomes as judged from the lack of any enhancement of the rate of NADH oxidation upon their addition to the suspension, neither did they affect the rate of pyruvate efflux induced by addition of acetoacetate.

Fig.2C shows the $acetoacetate_{in}/pyruvate_{out}$ exchange activity of proteoliposomes loaded with acetoacetate, the efflux of this being promoted by externally added pyruvate. It can be noted that pyruvate addition to the proteoliposome suspension caused, in contrast to what was observed for the $pyruvate_{in}/acetoacetate_{out}$ exchange, an immediate extrusion of acetoacetate, at a rate comparable to that attained by the acetoacetate-induced pyruvate efflux after the initial lag (see also table 1).

In order to determinate the kinetic constants of the pyruvate/acetoacetate exchange, the efflux of pyruvate from loaded proteoliposomes was measured for different concentrations of externally added acetoacetate.

Table 1

Substrate specificity and inhibitor sensitivity of the reconstituted monocarboxylate carrier

Substrate trapped into liposomes	Counteranion	Activity ($nmol \cdot min^{-1} \cdot mg^{-1}$ prot.)		
Pyruvate	Acetoacetate	1070	66	(3)
Pyruvate	Phosphate, malate, citrate		0	
Pyruvate + 2-cyano-4-hydroxycinnamate[a]	Acetoacetate	135	16	(3)
Acetoacetate	Pyruvate	1176	109	(3)

The HTP eluate was reconstituted in liposomes containing 200 mM of the indicated anions; 1.33 mM of the counteranions were added outside the proteoliposomes. The values of the activity, expressed as nmol anion exchanged, were corrected for the passive diffusion. Further details are given in the legend to fig.2 and in section 2. For each figure the mean value, SE and number of experiments are given

[a] Rat liver mithcondria were treated with 2 mM 2-cyano-4-hydroxycinnamate before solubilization (see text)

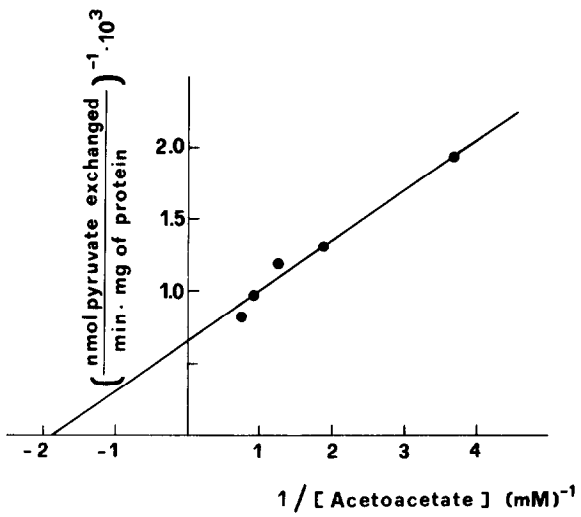

Fig.3. Double reciprocal plot of $pyruvate_{in}/acetoacetate_{out}$ exchange activity of the HTP eluate from Triton X-100 solubilized rat liver mitochondria reconstituted in liposomes. The rates of pyruvate/acetoacetate exchange were corrected for the net release of pyruvic acid from proteoliposomes. The line of best fit was obtained by the method of least-squares. The experimental conditions were those described in section 2 and in the legend to fig.2.

The data plotted according to Lineweaver-Burk give a straight line (fig.3). The values of K_m and V_{max} were 0.56 mM and 1560 nmol pyruvate exchanged per min and per mg of protein, respectively.

2-Cyano-4-hydroxycinnamate is an extremely potent inhibitor of the mitochondrial pyruvate translocator with which it forms a stable complex [19,20]. In table 1 the effect of this inhibitor on the exchange activity of the reconstituted system is reported. Because of the absorbance of 2-cyano-4-hydroxycinnamate at 360–374 nm, mitochondria were treated with 2 mM inhibitor before solubilization. It can be seen that the reaction was strongly inibited in proteoliposomes containing the eluate from 2-cyano-4-hydroxycinnamate-treated mitochondria.

4. CONCLUSIONS

The results presented show that the procedure used for the partial purification of an active monocarboxylate carrier from beef heart mitochondria can be satisfactorily applied to rat liver mitochondria. The only difference was the use of Triton X-100 instead of Triton X-114. Successful purification of the monocarboxylate carrier of rat liver mitochondria with the procedure used is documented by the following observations: (i) the HTP eluate reconstituted in liposomes catalyzes active $pyruvate_{in}/acetoacetate_{out}$ and $acetoacetate_{in}/pyruvate_{out}$ counter-exchange; (ii) the K_m value for pyruvate exchange is practically equal to that found in mitochondria [7]; (iii) the inhibitor sensitivity of the exchange reaction is similar to that of the mitochondrial pyruvate transporting system [19–22].

Taking into account the V_{max} value of the acetoacetate$_{in}$/pyruvate$_{out}$ exchange reaction measured in mitochondria amounting to 12 nmol/min/mg of protein [7], the purified carrier reconstituted in liposomes with a V_{max} of 1560 nmol/min/mg of protein was purified more than 100-fold.

The enzymatic method we have introduced to measure the activity of the monocarboxylate translocator makes it possible to follow the progress of the transport continuously and to determine its initial velocity. Interestingly, the kinetics pattern of the exchange activity seems to depend on which substrate was added inside and outside the vesicles (figs.2A and C). This behaviour requires further study. A tentative explanation could be that with pyruvate inside the vesicles in exchange with external acetoacetate, the carrier has to go through some activating turnovers before it reaches the maximal rate of exchange. This could be of physiological significance since in liver mitochondria the efflux of acetoacetate in exchange with cytosolic pyruvate has to be preferred to pyruvate$_{in}$/acetoacetate$_{out}$ exchange.

Acknowledgements: The invaluable advice of Dr Katarzina A. Nalecz and the skillful technical assistance of Mrs Anna Carlone are gratefully acknowledged.

REFERENCES

[1] Papa, S., Francavilla, A., Paradies, G. and Meduri, B. (1971) FEBS Lett. 12, 285–288.
[2] Papa, S. and Paradies, G. (1974) Eur. J. Biochem. 49, 265–274.
[3] Vaartijes, W.J., Geelen, M.J.H. and Van den Berg, 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. (1978) Biochem. J. 172, 377–387.
[7] Paradies, G. and Papa, S. (1978) in: Bioenergetics at Mitochondrial and Cellular Levels (Vojtczack, L., Lenartowicz, E. and Zborowski, J., eds) pp. 39–77, Nencki Institute of Experimental Biology, Warsaw.
[8] Thomas, A.P. and Halestrap, A.P. (1978) Biochem. J. 196, 471–479.
[9] Sherman, M.S. and Halestrap, A.P. (1984) Biochem. J. 223, 673–676.
[10] Azzi, A., Nalecz, M.J. and Wojtczak, L. (eds) (1989) Anion Carriers of Mitochondrial Membranes, Springer-Verlag, Berlin.
[11] Bolli, R., Nalecz, K.A. and Azzi, A. (1989) J. Biol. Chem. 264, 18024–18030.
[12] Bisaccia, F., Indiveri, C. and Palmieri, F. (1988) Biochim. Biophys. Acta 933, 229–240.
[13] Pedersen, P.L. (1977) Anal. Biochem. 80, 401–408.
[14] Laemmli, U.K. (1970) Nature 177, 680–685.
[15] De Pinto, V., Tommasino, M., Benz, R. and Palmieri, F. (1985) Biochim. Biophys. Acta 813, 230–242.
[16] Lowry, O.M., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[17] Nalecz, K.A., Bolli, R., Wojtczak, L. and Azzi, A. (1986) Biochim. Biophys. Acta 851, 29–37.
[18] Claeys, D., Muller, M. and Azzi, A. (1989) in: Anion Carriers of Mitochondrial Membranes (Azzi, A., Nalecz, K.A., Nalecz, M.J. and Wojtczak, L., eds) pp. 17–34, Springer-Verlag, Berlin.
[19] Halestrap, A.P. and Denton, R.N. (1974) Biochem. J. 138, 213–316.
[20] Thomas, A.P. and Halestrap, A.P. (1981) Biochem. J. 196, 471–479.
[21] Halestrap, A.P. (1975) Biochem. J. 148, 85–96.
[22] Paradies, G. and Papa, S. (1977) Biochim. Biophys. Acta 462, 333–346.