Volume 260, number 2, 217-219 FEB 08052 January 1990

Detection of the monocarboxylate transporter from pea mitochondria by means of a specific monoclonal antibody

Jeevalatha Vivekananda and David J. Oliver

Department of Bacteriology and Biochemistry, University of Idaho, Moscow, ID 83843, USA

Received 11 December 1989

The monocarboxylate (pyruvate) transporter from pea (*Pisum sativum*) mitochondria was identified by means of a specific monoclonal antibody. The antibody blocked pyruvate-dependent oxaloacetate metabolism without interfering with the metabolism of malate, α-ketoglutarate, or glycine. The antibody also blocked the pyruvate/pyruvate exchange reaction of the partially purified transporter reconstituted into phospholipid membranes. Using the specific monoclonal antibody, the transporter was identified on Western blots as a minor 19 kDa protein.

Pyruvate; Monocarboxylate; Transport; Mitochondria; Pea; Monoclonal antibody

1. INTRODUCTION

Substrates enter and leave the mitochondrial matrix via a family of specific transport proteins. These transporters are differentiated by their substrate specificities and inhibitor sensitivities [1]. The pyruvate transporter is responsible for connecting the glycolytic reactions in the cytosol with pyruvate dehydrogenase and ultimately the tricarboxylic acid cycle reactions of the mitochondrial matrix. This transporter has been studied from animal [2-5] and plant systems [6-8]. The transporter may work by either pyruvate/H^+ symport or pyruvate/OH^- antiport. The pyruvate carrier can transport pyruvate, lactate, and acetoacetate and is sensitive to inhibition by sulfhydryl reagents, particularly the α-cyanocinnamate derivatives [3].

Our laboratory has recently developed a procedure that allows us to isolate monoclonal antibodies that specifically bind to and inactivate individual mitochondrial substrate transporters. This procedure has been used to identify the dicarboxylate [10] and the glutamate/aspartate transporters [11] from pea mitochondria. In this paper we report the isolation of a monoclonal antibody that binds specifically to the monocarboxylate transporter from pea mitochondria and the use of the antibody to identify this transport protein on Western blots.

2. MATERIALS AND METHODS

Monoclonal antibodies were generated against mitochondrial membrane proteins as described [10,11]. The antibodies specific for the monocarboxylate transporter were selected by screening the resulting monoclonal library for antibodies that would inhibit pyruvate-dependent [4-^{14}C]OAA (oxaloacetate) metabolism in pea mitoplasts. The mitochondria were suspended in a reaction mixture containing 0.3 M sorbitol, 20 mM MOPS, 10 mM KPi, 1 mM EDTA, 0.1 mM coenzyme A, and 0.5 mM TPP (pH 7.2) and incubated with the supernatants of the hybridoma cultures. Following 60 min at 0°C to allow for antibody binding to the transporter, 10 mM pyruvate was added to the antibody-treated mitochondria and the mitochondria were allowed to metabolize the substrate at room temperature for 3 min before 0.5 mM [4-^{14}C]OAA was added. After 1 min the reaction was terminated with 1% perchloric acid and unreacted [4-^{14}C]OAA was decarboxylated by reaction with $CuSO_4$ [12,13]. Any labelled OAA that had been reduced to malate, dependent on NADH produced by pyruvate oxidation, or condensed with acetyl CoA, derived from pyruvate decarboxylation, was not affected by the copper treatment and could be quantified by liquid scintillation counting. Antibodies that inactivated the pyruvate transporter were detected by their ability to specifically inhibit pyruvate-dependent OAA metabolism.

The monocarboxylate transporter was solubilized from pea mitochondria in 3% Triton X-114 containing 1 mg/ml cardiolipin, partially purified by passing the extract over a hydroxylapatite column, and incorporated into asolectin vesicles [10,14]. The proteoliposomes were preloaded with 6 mM pyruvate and the α-cyano-4-hydroxycinnamate-sensitive exchange for external [^{14}C]pyruvate measured [10,14-16]. The effect of the antibody on the reconstituted transport reaction was determined by preincubating the proteoliposome with hybridoma supernatant for 1 h before determining the exchange rate.

3. RESULTS AND DISCUSSION

Two monoclonal antibodies, labelled AB17 and AB30, were identified that specifically inhibited OAA metabolism linked to pyruvate (table 1). Given that the antibodies reacted only with antigens exposed to the external surface of the inner mitochondrial membranes and that the assay did not rely on the electron transport chain, the only known targets for antibody binding were the substrate transporters. The specificity of the

Correspondence address: D.J. Oliver, Department of Bacteriology and Biochemistry, University of Idaho, Moscow, ID 83843, USA

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

Table 1

Effect of monoclonal antibodies AB17 and AB30 on substrate-linked OAA metabolism by pea mitochondria

Substrate	Rate of oxaloacetate metabolism (nmol/mg protein/min)		
	Control	+AB17	+AB30
α-Ketoglutarate	92	94 (102%)	90 (98%)
Glycine	329	345 (104%)	343 (105%)
Malate	378	403 (107%)	385 (102%)
Pyruvate	303	208 (69%)	236 (78%)

[4-^{14}C]Oxaloacetate metabolism linked to the metabolism of the different substrates was measured in pea mitochondria. Where indicated the mitochondria were incubated with one of the monoclonal antibodies before the assay period

inhibition was taken as evidence that the antibodies reacted with the monocarboxylate transporter and blocked its function. The maximum inhibition of pyruvate metabolism was approximately 30%. This limited amount of inhibition probably resulted from the fact that only a portion of the outer membrane had been removed [10] and as a result some portion of the monocarboxylate transporter population was protected from the antibody.

Although the metabolic assay system described allowed for the rapid screening of a large number of antibodies, the assays are indirect. Conclusive proof that the antibodies were specifically targeting the monocarboxylate transporter was provided by experiments where the partially purified transporter fraction was reconstituted into phospholipid vesicles and the effect of the antibody on pyruvate/pyruvate exchange was

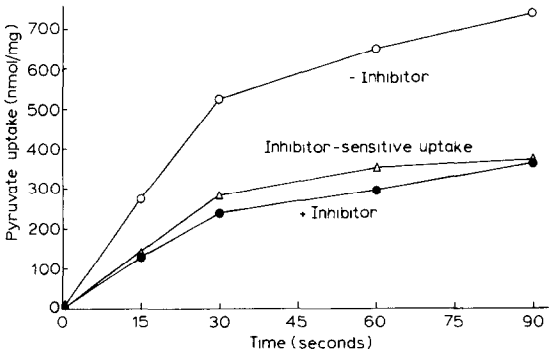

Fig.1. Pyruvate uptake by phospholipid vesicles reconstituted with the monocarboxylate transporter. The vesicles were preloaded with 6 mM pyruvate and the external pyruvate removed by chromatography on Dowex AG1-X8. The vesicles were then exposed to 1 mM [^{14}C] pyruvate for the time indicated before the reaction was terminated by the addition of 2 mM α-cyano-4-hydroxycinnamate and the incorporated radioisotope separated on a spun Sephadex G50 column. The (+) inhibitor samples were pretreated with 1 mM α-cyano-4-hydroxycinnamate for 3 min before the addition of the labelled pyruvate. The inhibitor-sensitive transport was the difference between the (+) inhibitor and (−) inhibitor samples and was taken to represent transport through the monocarboxylate transporter.

Table 2

Inhibition of pyruvate/pyruvate exchange wth the reconstituted monocarboxylate transporter by the monoclonal antibodies AB17 and AB30

Condition	Pyruvate exchange	
	(nmol/mg protein/min)	(%)
Control	313	
+ hybridoma supernatant	280	100%
+ antibody AB17	100	36%
+ antibody AB30	79	28%

The hydroxylapatite fraction of pea mitochondria was reconstituted into asolectin vesicles that were then preloaded with unlabelled pyruvate. Following a 60 min incubation with either a control hybridoma supernatant or a supernatant from one of the antibody-producing cultures, the rate of exchange for external [^{14}C]pyruvate was measured [10,14]

measured. Fig.1 shows the kinetics for the exchange of internal unlabelled pyruvate for external ^{14}C-pyruvate by proteolipid vesicles that contained the purified transporter fraction. In order to determine the rate of specific pyruvate/pyruvate exchange through the monocarboxylate transporter, measurements were done in parallel with two proteoliposome samples, one of

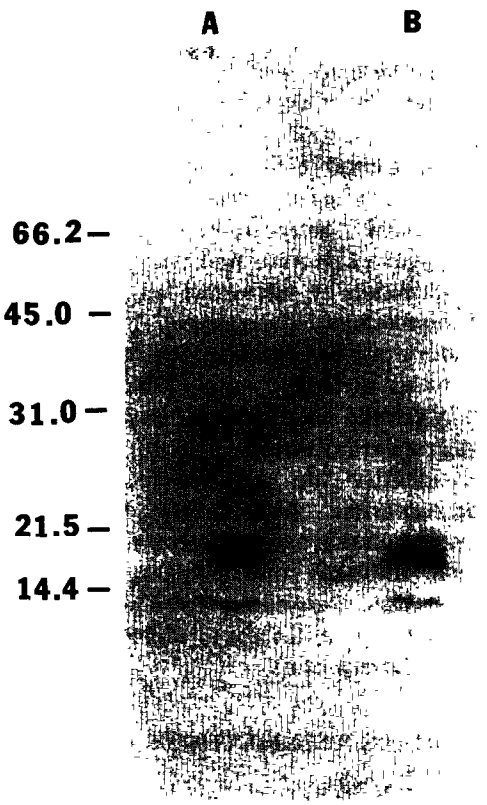

Fig.2. Identification of the monocarboxylate transporter on a Western blot of crude pea mitochondria and the hydroxylapatite fraction using monoclonal antibody AB17. Lane A contained 0.5 mg of total mitochondrial protein and lane B contained 10 μg of protein from the hydroxylapatite fraction.

which was pretreated with 1 mM α-cyano-4-hydroxycinnamate. The rate of inhibitor-sensitive transport was linear for less than 30 s and was at least 500 nmol pyruvate exchanged (mg protein^{-1} min^{-1}).

Both monoclonal antibodies inhibited the rate of α-cyano-4-hydroxycinnamate-sensitive pyruvate/pyruvate exchange by the monocarboxylate transporter reconstituted into phospholipid vesicles (table 2). The inhibition was approximately 70% complete. Because the transport reaction in liposomes was exclusively dependent on the monocarboxylate transporter, this inhibition provided very strong evidence that these monoclonal antibodies were specifically binding to that transport protein.

The antibodies were then used to identify the specific protein(s) involved. The Western blots for antibody AB30 revealed at least 5 bands suggesting that while the antibody was binding to the monocarboxylate transporter, it was also recognizing a number of other epitopes in mitochondria. Monoclonal antibody AB17, however, was much more specific and bound to a single major protein (fig.2). The size of the monocarboxylate transporter was 19 kDa as determined by SDS-PAGE.

4. CONCLUSIONS

The monocarboxylate transporter is located in the mitochondrial inner membrane and has a key function in connecting glycolysis in the cytosol with pyruvate dehydrogenase and the tricarboxylic acid cycle in the mitochondrial matrix. Like all of the mitochondrial substrate transporters it is extremely hydrophobic and can only be manipulated in detergent micelles. The three major transporters associated with energy conservation, the adenylate transporter, the phosphate transporter, and the uncoupler protein of brown adipose tissue, are present at high concentrations in the mitochondrial membrane and have been purified to near homogeneity. The minor substrate transporters, including the monocarboxylate, dicarboxylate, tricarboxylate, and α-ketoglutarate transporters, are present at much lower levels and have not yet been completely purified. By taking advantage of the ability of monoclonal antibody techniques to obtain specific monoclonal antibodies without first obtaining pure antigens, we have been able to screen for antibodies that will react specifically with the dicarboxylate [10], glutamate/aspartate [11], and now the monocarboxylate transporters. We are currently using these antibodies to screen for cDNA clones for these transporters in order to continue structural analyses.

The monocarboxylate transporter identified by AB17 has an apparent molecular mass of 19 kDa. This is in reasonably good agreement with the observations of Thomas and Halestrap [9] that a 15 kDa protein from rat liver and heart mitochondria was labelled by [^{3}H]*N*-phenylmaleimide in a manner that paralleled inhibition of the monocarboxylate transporter. This transporter appeared to be smaller than the other substrate transporters identified to date. The dicarboxylate transporter from peas has a molecular mass of 26 kDa [10] and the glutamate/aspartate transporter from the same source was 21 kDa [11]. The adenylate and phosphate transporters and the uncoupler protein have molecular masses in the range of 33–35 kDa (for a review see [18]). There is no obvious reason for the smaller size of the monocarboxylate transporter particularly considering how similar its reaction is to that of the phosphate transporter. The adenylate carrier and the uncoupler protein are both homodimers [17] and this is generally accepted to be true for the other mitochondrial transport proteins. Should this also hold for the monocarboxylate transporter, then the 19 kDa polypeptide identified is the only subunit of this carrier. It is possible, however, that this is only one subunit of a multiple subunit protein.

The use of monoclonal antibodies has allowed us to identify three minor substrate transporters in pea mitochondria. This technique may prove equally useful for identifying other membrane proteins that have resisted conventional purification techniques where rapid screening systems can be devised.

REFERENCES

[1] LaNoue, D.F. and Schoolwerth, A.C. (1979) Annu. Rev. Biochem. 48, 871–922.
[2] Papa, S., Francavilla, A., Paradies, G. and Meduri, B. (1971) FEBS Lett. 12, 285–288.
[3] Halestrap, A.P. and Denton, R.M. (1974) Biochem. J. 138, 313–316.
[4] Paradies, G. and Papa, S. (1977) Biochim. Biophys. Acta 462, 333–346.
[5] Halestrap, A.P. (1978) Biochem. J. 172, 377–387.
[6] Day, D.A. and Hanson, J.B. (1977) Plant Physiol. 59, 630–635.
[7] Proudlove, M.O. and Moore, A.L. (1982) FEBS Lett. 147, 26–30.
[8] Brailsford, M.A., Thompson, A.G., Kaderbhai, N. and Beechey, R.B. (1986) Biochem. J. 239, 355–361.
[9] Thomas, A.P. and Halestrap, A.P. (1981) Biochem. J. 196, 471–479.
[10] Vivekananda, J., Beck, C.F. and Oliver, D.J. (1988) J. Biol. Chem. 263, 4782–4788.
[11] Vivekananda, J. and Oliver, D.J. (1989) Plant. Physiol. 91, 272–277.
[12] Ebbighausen, H., Jia, C. and Heldt, H.W. (1985) Biochim. Biophys. Acta 810, 184–199.
[13] Hatch, M.D. and Heldt, H.W. (1985) Anal. Biochem. 145, 393–397.
[14] Kaplan, R.S. and Pederson, P.L. (1985) J. Biol. Chem. 260, 10293–10298.
[15] Nalecz, M.J., Nalecz, D.A., Broger, C., Bolli, R., Wojtczak, L. and Azzi, A. (1986) FEBS Lett. 196, 331–336.
[16] Nalecz, K.A., Bolli, R., Wojtczak, L. and Azzi, A. (1986) Biochim. Biophys. Acta 851, 29–37.
[17] Aquila, H., Link, T.A. and Klingenberg, M. (1987) FEBS Lett. 212, 1–9.