THE SEPARATION OF DPN-LINKED AND TPN-LINKED ISOCITRATE DEHYDROGENASE ACTIVITIES OF MAMMALIAN LIVER

G. W. E. Plaut and T. Aogaichi

Department of Biochemistry, Rutgers Medical School, Rutgers . The State University, New Brunswick, New Jersey

Received July 24, 1967

The mechanism of isocitrate oxidation by liver mitochondria has been studied by several investigators, in particular to determine whether substrate dehydrogenation occurs by way of the TPN-linked or the DPN-linked isocitrate dehydrogenases (for review see Plaut (1963)).

Initial studies of DPN-linked isocitrate dehydrogenase from animal tissues demonstrated the activity in extracts of acetone powders of mitochondria from heart, pigeon breast muscle and kidney. The TPN- and DPN-linked dehydrogenases were separated in the cases of heart, pigeon breast muscle (Plaut and Sung, 1954), and human placenta (Sung and Hsu, 1957). However, the reduction of DPN by isocitrate was very slow with preparations from liver mitochondria although relatively high activity for the corresponding TPN-linked enzyme was found. These experiments thus did not prove the occurrence of the DPN enzyme in liver.

The finding that DPN-linked enzyme from heart is activated by ADP (Chen and Plaut, 1963) prompted reinvestigation of the levels of this enzyme in various tissues including liver (Goebell and Klingenberg, 1964; Stein et al., 1967). However, the measurement of the DPN enzyme at 340 mμ in the presence

of ADP presents certain complications in extracts from tissues such as liver, where the activity is low compared to that of the corresponding TPN-enzyme. Liver mitochondria contain active adenylate kinases (Chiga and Plaut, 1960; Chiga et al., 1961; Markland and Wadkins, 1966) and the phosphorylation of DPN to TPN by ATP with subsequent oxidation of isocitrate by the TPN-linked isocitrate dehydrogenase has been observed (Vignais and Vignais, 1961).

The present work resolves uncertainties about the actual occurrence of DPN-linked isocitrate dehydrogenase in liver, since a complete separation of the DPN- and TPN-linked enzyme was achieved, indicating that in this as in other tissues these activities are associated with separate protein entities.

MATERIALS AND METHODS

The reagents, assay procedures and units used were as described by Chen and Plaut (1963) for the enzyme from heart.

PURIFICATION

Rat Liver Enzyme

Extract - Mitochondria from 48 g of rat liver (Schneider, 1957) were suspended in 105 ml of potassium phosphate (89.5 mM)-EDTA (1.8 mM)-red. glutathione (9 mM)-ADP (0.9 mM) at pH 7.2. The suspension was homogenized for three 30 sec periods in a Virtis 45 apparatus at full speed. Particles were removed by sequential centrifugation for 30 min periods in the Sorvall RC2 and Spinco L centrifuges at 15,000 and 40,000 RPM, respectively. Subsequent operations were carried out at room temperature.

$(NH_4)_2SO_4$ and heat steps - The supernatant fluid was brought to 50% sat. with solid $(NH_4)_2SO_4$ and the residue was

taken up in 25 ml of 30% sat. $(NH_4)_2SO_4$ at pH 7.0. The solution was heated at 50° for 15 min, cooled to 25° and centrifuged.

Second $(NH_4)_2SO_4$ fractionation - The supernatant was brought to 50% sat. with $(NH_4)_2SO_4$. The residue was dissolved in 5 ml of 30% sat. $(NH_4)_2SO_4$ at pH 7.0. This fraction contained a major part of the DPN-linked enzyme, but very little TPN-isocitrate dehydrogenase activity.

Rabbit Liver Enzyme

Extract, heat step and $(NH_4)_2SO_4$ fractionation - Particles prepared from 790 g of rabbit liver (Plaut, 1957), were suspended in about 1 L of $(NH_4)_2SO_4$-buffer[1] and treated in a Virtis homogenizer. The residue was removed by centrifugation and the supernatant fluid was brought to 50° for 15 min then cooled to room temperature.

First $(NH_4)_2SO_4$ fractionation - The $(NH_4)_2SO_4$ concentration of the clarified fluid was raised to 60% sat. with solid salt. The precipitate was dissolved in $(NH_4)_2SO_4$-buffer[1]. After clarification the solution was dialyzed overnight against 20 vol of $(NH_4)_2SO_4$-buffer[1].

Second $(NH_4)_2SO_4$ fractionation - The solution was brought to 55% sat. with sat. $(NH_4)_2SO_4$ and the residue was taken up in 20 ml of $(NH_4)_2SO_4$-buffer[1].

Sephadex chromatography - Columns of Sephadex G-100, 2.5 cm x 40 cm, were equilibrated with $(NH_4)_2SO_4$-buffer. 2 ml portions of the enzyme solution were applied. The buffer was passed through the column at 0.2 ml per min and 2 ml fractions were collected.

Third $(NH_4)_2SO_4$ fractionation - Fractions containing the

[1] 30% sat. $(NH_4)_2SO_4$-10 mM potassium phosphate-2 mM EDTA (pH 7.0).

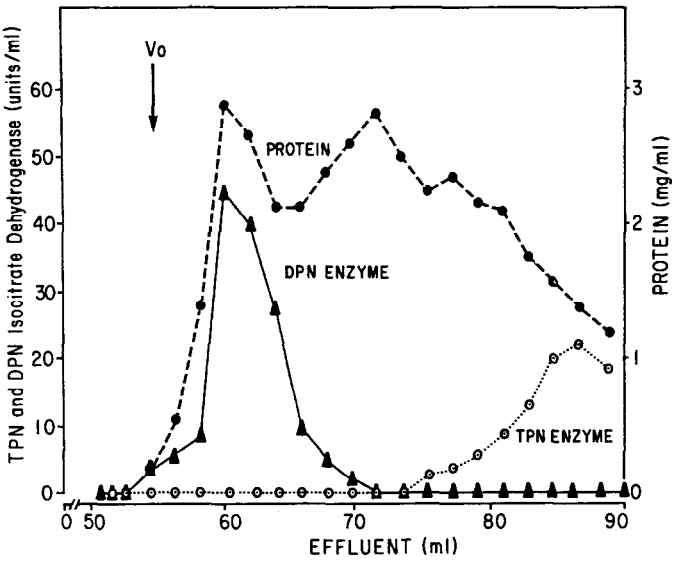

Fig. 1. Separation of DPN- and TPN-linked isocitrate dehydrogenases on Sephadex G-100. Experimental details are given in the text.

DPN-linked enzyme (Fig. 1) were combined and dialyzed for 1 hr against 10 vol of 40% sat. $(NH_4)_2SO_4$-1 mM EDTA at pH 7.0, then against 42% sat. $(NH_4)_2SO_4$-1 mM EDTA at pH 7.0 for 2 hrs. The clarified solution was adjusted to 55% sat. with sat. $(NH_4)_2SO_4$ and the precipitate was dissolved in 7 ml of $(NH_4)_2SO_4$-buffer.

Fourth $(NH_4)_2SO_4$ fractionation - The solution was dialyzed overnight against 500 ml of 40% sat. $(NH_4)_2SO_4$-1 mM EDTA at pH 7.0 and acidified to pH 5.5 with 0.3 ml of 1 M KH_2PO_4. The $(NH_4)_2SO_4$ concentration was adjusted to 42% sat., inert protein was removed, and the supernatant was brought to 54% sat. with sat. $(NH_4)_2SO_4$. The precipitate was collected, dissolved in 1 ml of 1.2 M Na_2SO_4-1 mM EDTA-10 mM imidazole HCl at pH 7.0, and dialyzed overnight against 100 ml of the 1.2 M Na_2SO_4-buffer.

Table I

Separation of DPN- and TPN-linked isocitrate dehydrogenases from rat and rabbit liver

	DPN isocitrate dehydrogenase				TPN isocitrate dehydrogenase
Rat Liver (48 g)					
Treatment	Vol (ml)	Protein (mg)	Activity (units)	Specific activity (u./mg)	Activity (units)
Extract	96	395	1250	3.2	6630
1st $(NH_4)_2SO_4$ and heat step	25	78	417	5.4	125
2nd $(NH_4)_2SO_4$					
0.3-0.5 sat.	9	46	409	8.9	0
0.5-0.6 sat.	4	32	0	0	106
Rabbit Liver (790 g)					
Extract	1147	6060	8100	1.34	94,200
Heat step	1098	4112	5500	1.33	93,300
1st $(NH_4)_2SO_4$ fractionation	71	1625	5700	3.50	14,000
2nd $(NH_4)_2SO_4$ fractionation	20	1085	5500	5.1	6,300
Sephadex chromatography	176	202	1840	9.1	0
3rd $(NH_4)_2SO_4$ fractionation	6.5	28	419	15	-
4th $(NH_4)_2SO_4$ fractionation	1.0	9.8	368	38	0

The fractionation procedures have been summarized in Table I.

RESULTS AND DISCUSSION

Extraction and stability - DPN enzyme activity from liver exhibited marked cold lability during the early stages of preparation. Thus, none of the TPN enzyme, but most of the DPN enzyme of extracts was lost upon storage overnight in an ice bath; little loss in activity was encountered when stored at 25° for 4 days in solutions of high electrolyte content, e.g., 1.2 M Na_2SO_4 or 30% sat. $(NH_4)_2SO_4$. This property may

account in part for the low yield of DPN enzyme in extracts of acetone desiccated (Plaut and Sung, 1954) or lyophilized mitochondria.

Separation of enzyme activities - Separation of the DPN- and TPN-linked isocitrate dehydrogenase from mitochondria of rat and rabbit liver has been achieved by the methods outlined in Table I which differ in certain details. In the case of rat liver the mitochondria were disintegrated in a relatively dilute phosphate buffer while with rabbit liver the activity was extracted with a medium of high electrolyte concentration. Although the procedure outlined for rat liver leads to a separation of the DPN- and TPN-linked enzymes at an early stage, the method described for rabbit liver offers certain advantages. The high speed centrifugation steps (rat liver procedure) are not necessary in the rabbit liver preparation, and since the DPN enzyme is rather unstable in solutions of dilute electrolytes, the latter preparation gives more consistent results.

A complete separation of the DPN and TPN enzymes can be accomplished by chromatography on Sephadex G-100. The DPN enzyme emerges from the column at the void volume (V_o)[2] while the TPN-linked isocitrate dehydrogenase is retarded considerably (Fig. 1). These results are in agreement with studies on the enzyme from heart, in which it was found that the DPN enzyme is a much larger molecule than the TPN-linked dehydrogenase (Chen and Plaut, 1963; Chen et al., 1964).

The final product obtained in either procedure is free from glutamate dehydrogenase activity.

[2] For the purpose of the present operation V_o is defined as the volume of effluent collected up to the point where Dextran Blue first emerged from the column.

Properties - Under the conditions of the assay the DPN enzyme has maximal activity between pH 7.2-7.7.

Preliminary results indicate that the DPN enzyme from liver, as that from heart (Chen and Plaut, 1963), is activated by ADP and inhibited by DPNH and ATP. TPNH potentiates the inhibition by DPNH. Mn^{++} or Mg^{++} is required for activity.

ACKNOWLEDGEMENT

This work was supported in part by grant AM 10501 from the NIAMD, NIH, U.S. Public Health Service.

REFERENCES

Chen, R. F., Brown, D. M., and Plaut, G. W. E., Biochemistry 3, 552 (1964).

Chen, R. F. and Plaut, G. W. E., Biochemistry 2, 1023 (1963).

Chiga, M. and Plaut, G. W. E., J. Biol. Chem. 235, 3260 (1960).

Chiga, M., Rogers, A. E., and Plaut, G. W. E., J. Biol. Chem. 236, 1800 (1961).

Goebell, H. and Klingenberg, M., Biochem. Z., 340, 441 (1964).

Markland, F. S. and Wadkins, C. L., J. Biol. Chem., 241, 4124 (1966).

Plaut, G. W. E., Arch. Biochem. Biophys. 69, 320 (1957).

Plaut, G. W. E. in P. D. Boyer, H. Lardy, and K. Myrbäck, The Enzymes, 2nd ed., Vol. 7, Academic Press, New York, 1963, p. 105.

Plaut, G. W. E. and Sung, S.-C., J. Biol. Chem., 207, 305 (1954)

Schneider, W. C. in W. W. Umbreit, R. H. Burris, and J. F. Stauffer, Manometric Techniques, 3rd ed., Burgess Publishing Company, Minneapolis, 1957, p. 188.

Stein, A. M., Stein, J. H., and Kirkman, S. K., Biochemistry, 6, 1370 (1967).

Sung, S.-C. and Hsu, C. H., Formosan Med. Ass'n. 56, 103 (1957).

Vignais, P. V. and Vignais, P. M., Biochim. Biophys. Acta 47, 515 (1961).