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CRYSTALLIZATION AND PROPERTIES OF RAT LIVER MALATE DEHYDROGENASE (DECARBOXYLATING) ($NADP^+$)

F. WADA, N. NUMATA, Y. EGUCHI and Y. SAKAMOTO

Institute for Cancer Research, Osaka University Medical School, Osaka, 553 (Japan)

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Summary

Rat liver malate dehydrogenase (decarboxylating) ($NADP^+$) (L-malate: $NADP^+$ oxidoreductase (oxaloacetate-decarboxylating), EC 1.1.1.40) was purified and crystallized from medium containing 30 mM Tris · HCl buffer (pH 7.7), 5 mM $MgCl_2$ and 2 mM 2-mercaptoethanol. The enzyme formed rhomboid crystals free from coenzyme, and appeared homogeneous on isoelectric focusing. The crystalline enzyme had an isoelectric point of pH 6.3. Amino acid analysis showed that it contained more acidic amino acids than basic ones.

Introduction

Malate dehydrogenase (decarboxylating) ($NADP^+$) (L-malate: $NADP^+$ oxidoreductase (oxaloacetate-decarboxylating), EC 1.1.1.40) was crystallized as its $NADP^+$ complex from the supernatant fraction of pigeon liver by Hsu and Lardy [1]. Several workers [2—5] purified the enzyme from rat liver, but could not crystallize it. This paper reports a procedure for crystallization of coenzyme-free malate dehydrogenase from the supernatant fraction of rat liver and some properties of the crystalline enzyme.

Materials and Methods

Enzyme activity was assayed at 25°C essentially by the method of Ochoa et al. [6]. The reaction mixture consisted of 100 mM Tris · HCl (pH 7.4) 5 mM $MgCl_2$, 0.5 mM $NADP^+$, 3 mM potassium L-malate and enzyme in a total volume of 1.0 ml. A unit of enzyme activity is defined as 1 μmol of $NADP^+$ reduced per min. Protein was determined by the biuret method [7] with crystalline bovine serum albumin as the standard.

Sprague-Dawley rats, weighing 200—250 g, were starved for 3 days and

then given a high carbohydrate, low fat diet containing 1% thyroid powder for 3 days before sacrifice, to induce the enzyme [2].

In this work malate dehydrogenase was purified from the supernatant fraction of rat liver by modifications of the method of Isohashi et al. [2]. The following modifications were made. The livers (about 700 g) were homogenized with 1 vol. of medium (pH 7.4) containing 0.25 M sucrose, 5 mM $MgCl_2$, 1 mM EDTA, and 2-mercaptoethanol and the homogenate was centrifuged at 10 000 × *g*, for 20 min. The resulting supernatant fluid was subjected to heat treatment. The preparation was fractionated with 60—75% saturation of ammonium sulfate and 23—43% ethanol. DEAE-cellulose chromatography was carried out at pH 7.7. Fractions with the same specific activity were combined and concentrated by ultrafiltration. At a protein concentration of more than 1 mg per ml of 30 mM Tris · HCl buffer (pH 7.7) containing 2 mM 2-mercaptoethanol and 5 mM $MgCl_2$, a sheen of crystals appeared after a few hours. Usually, crystallization occurred in fractions of eluate during DEAE-cellulose chromatography. The crystals were rather insoluble in 30 mM Tris · HCl buffer, but were soluble in high concentrations of buffers and salts, e.g. 2 mg of protein were soluble per ml of 250 mM potassium phosphate or Tris buffer. 9 vol. of saturated ammonium sulfate solution were added gradually to the crystalline suspension in 30 mM Tris · HCl buffer (4 mg/ml). During the addition, crystals of the malate dehydrogenase dissolved and then the enzyme protein was precipitated by salting out. The precipitate was dissolved in 30 mM Tris · HCl buffer to give a concentration of 2 mg/ml and dialyzed against 30 mM Tris · HCl buffer (pH 7.7) containing 5 mM $MgCl_2$ and 2 mM 2-mercaptoethanol. Recrystallization was accomplished by dialysis for one day.

Isoelectric focusing on polyacrylamide gel containing Ampholine (pH range 3.5—10) was performed according to the method of Righetti and Drysdale [8]. The gels were stained for protein with Coomassie Blue and the enzyme activity was demonstrated by similar gels in a staining mixture (pH 7.4) containing 10 mM potassium L-malate, 1 mM $NADP^+$, 5 mM $MgCl_2$, 0.1 M Tris HCl buffer, 0.25 mg/ml nitroblue tetrazolium and 0.25 mg/ml phenazine methosulfate.

Amino acid analyses were performed on a Hitachi KLA-3B amino acid analyzer.

Results and Discussion

Mg^{2+} was essential for crystallization but 2-mercaptoethanol was not. Recrystallization occurred at both pH 7.7 and 7.4. Therefore, a small pH change (from 7.4 to 7.7) during DEAE-cellulose chromatography might not be responsible for success in crystallization. Murphy and Walker [4] could not crystallize the enzyme at pH 7.7. The reasons for the facilitated crystallization may be differences in the treatment of the enzyme during purification and yield. A typical protocol for crystallization is shown on Table I, and a photomicrograph of these crystals is shown in Fig. 1. Samples of the purified enzyme gave a single band that stained for both protein and enzyme activity on isoelectric focusing. Li et al. [5] observed three bands on polyacrylamide gel electrophoresis and attributed them to isoenzymes. We also observed three bands that

TABLE I

PURIFICATION OF RAT LIVER MALATE DEHYDROGENASE

A unit of enzyme activity is defined as 1 μmol $NADP^+$ reduced per min.

Fraction	Vol. (ml)	Total protein (mg)	Specific activity (units/mg)	Purification (-fold)	Total activity (units)	Yield (%)
Supernatant fraction	650	58 500	0.08	1	4680	100
Acid and heat treatment	480	23 520	0.16	2	3763	80
Ammonium sulfate fractionation	30	990	2.88	36	2851	61
Sephadex G-200 filtration	70	733	3.68	46	2679	58
Ethanol fractionation	30	227	8.02	100	1820	39
DEAE-cellulose chromatography	30	34	41.0	513	1394	30
Crystallization	5	26	41.0	513	1066	23
Recrystallization	5	18	41.0	513	738	16

stained for both protein and enzyme activity by inappropriate treatment of the cyrstalline enzyme. Murphy and Walker [4] reported similar results. The isoelectric point of the enzyme was pH 6.3 (Fig. 2). Fig. 3 shows the ultraviolet-absorption spectrum of the purified malate dehydrogenase. The extinction of a 0.1% solution of crystalline malate dehydrogenase at 287 nm was calculated to be 0.72. The crystalline enzyme was free of $NADP^+$, because addition of malate to the enzyme solution caused no increase in absorption at 340 nm. The amino acid composition of the crystalline enzyme (Table II) was similar to that of the purified (not crystallized) enzyme reported by Li et al. [5]. The acidic isoelectric point (6.3) is attributed to the predominance of acidic amino acids (gluta-

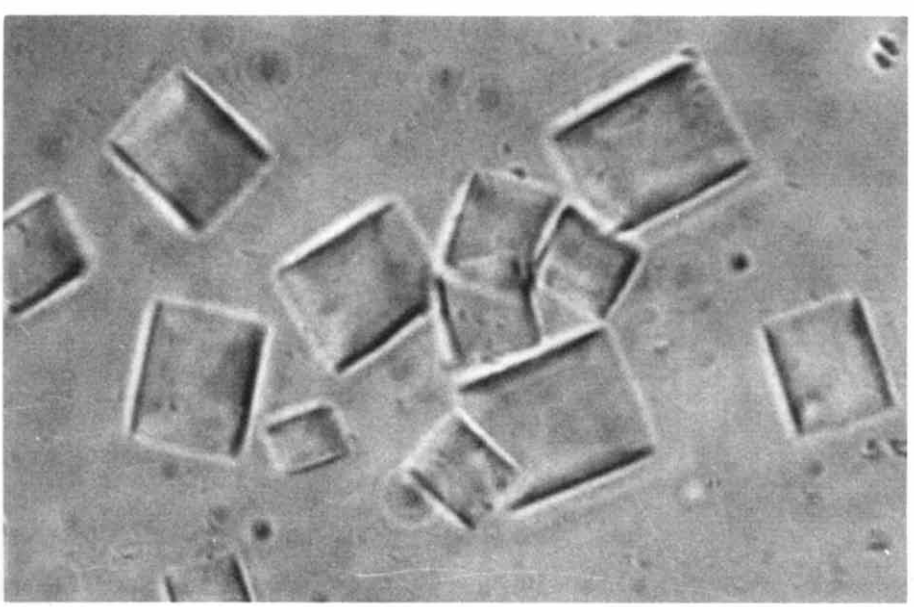

Fig. 1. Crystalline malate dehydrogenase from rat liver at 300X magnification.

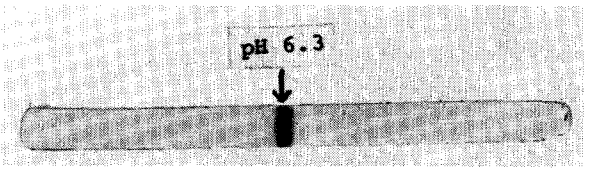

Fig. 2. Isoelectric focusing of crystalline malate dehydrogenase (100 μg) on 4% polyacrylamide containing 3.2% Ampholine (pH range 3.5—10). A current of 1 mA for 3 h was used and the gel was stained with Coomassie Blue. The pH at left end was 8.8 and right 3.9.

mic acid + aspartic acid = 487) over basic amino acids (lysine + histidine + arginine = 305).

Several workers have used 2-mercaptoethanol as a stabilizer during purification of malate dehydrogenase [1—5]. However, Saito et al. [3] reported that the purified enzyme was unstable in its presence. The reason for this inconsistency was the absence or presence of Mg^{2+}. Addition of 2-mercaptoethanol protected the enzyme from inactivation in the presence of Mg^{2+}, but increased

TABLE II

AMINO ACID COMPOSITION OF RAT LIVER MALATE DEHYDROGENASE

The sample contained 1.53% ash. It was hydrolyzed in 4 M methanesulfonic acid containing 0.2% tryptamine hydrochloride.

Amino acid	Amino acid residues (g/100 g protein)					Mol of residues per 268 000 g (67 000 g) [5]
	Hydrolysis				Average X 1/0.9515 (Value corrected for water)	
	24 h	48 h	72 h	Average		
Lys	6.25	6.52	6.41	6.40	6.83	143 (36)
His	2.09	2.17	2.17	2.14	2.25	44 (11)
Arg	6.45	6.59	6.52	6.52	6.85	118 (29)
Asp	9.33	9.56	9.32	9.40	9.88	230 (58)
Thr	3.50	3.52	3.34	3.61*	3.79	101 (25)
Ser	3.09	2.88	2.48	3.38*	3.55	109 (27)
Glu	11.54	11.89	11.89	11.77	12.37	257 (64)
Pro	4.12	4.01	4.03	4.05	4.26	118 (29)
Gly	3.04	3.21	3.17	3.14	3.30	155 (39)
Ala	4.40	4.72	4.56	4.56	4.79	181 (45)
Val	4.95	5.45	5.75	5.75**	6.04	163 (41)
Met	2.20	2.28	2.30	2.26	2.38	49 (12)
Ile	4.60	5.16	5.28	5.28**	5.55	114 (28)
Leu	9.88	9.59	9.66	9.69	10.18	208 (52)
Tyr	4.39	4.25	4.24	4.29	4.51	74 (19)
Phe	4.90	4.96	4.91	4.92	5.17	94 (24)
Half-Cys***	—	—	—			
Trp	0.57	0.86	0.68	0.70	0.74	11 (3)
Water	4.85	4.85	4.85			
Total	90.15	95.75	91.56			

* Values extrapolated to zero time.
** Values after 72 h hydrolysis.
*** Not determined.

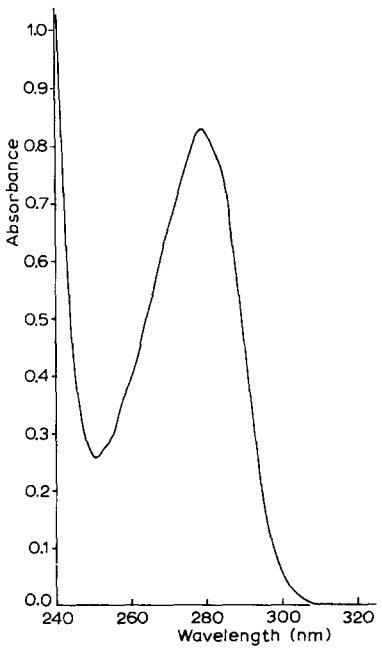

Fig. 3. Ultraviolet-absorption spectrum of crystalline malate dehydrogenase. The sample cuvette contained 1.15 mg of crystalline enzyme per ml of 0.1 M potassium phosphate buffer (pH 7.4) and the blank contained buffer only.

its inactivation in the absence of Mg^{2+} (Table III). Glutathione, a physiological SH-reagent, was more potent than 2-mercaptoethanol in the inactivation of the enzyme in the absence of Mg^{2+}.

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TABLE III

EFFECT OF 2-MERCAPTOETHANOL ON THE STABILITY OF PURIFIED ENZYME IN THE ABSENCE OR PRESENCE OF Mg^{2+}

Enzyme solution (0.01 mg per ml of 50 mM Tris · HCl buffer, pH 7.4) was stored at 0°C.

	Initial	1 day	4 days
No addition	100	72	65
Mercaptoethanol (2 mM)	100	60	44
$MgCl_2$ (5 mM)	100	76	67
$MgCl_2$ (5 mM) + mercaptoethanol (2 mM)	100	80	79

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