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Gian Ludovico Melzi D'Eril, Remigio Moratti, Augusto Montani, Rosa Pasquinelli, Maria Assunta Santacroce

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Biochimica. — *Simultaneous purification of aconitate hydratase and isocitrate* (NADP) *dehydrogenase from mitochondria.* Nota di GIAN LUDOVICO MELZI D'ERIL (*), REMIGIO MORATTI (*), AUGUSTO MONTANI (*), ROSA PASQUINELLI (**) e MARIA ASSUNTA SANTACROCE (**), presentata (***) dal Corrisp. A. RUFFO.

RIASSUNTO. — Viene descritto un metodo che permette la purificazione contemporanea di aconitato idratasi ed isocitrato(NADP)deidrogenasi partendo da una singola preparazione di mitocondri di cuore di vitello. L'estratto mitocondriale è stato precipitato con $(NH_4)_2SO_4$ e gli enzimi ivi contenuti purificati tramite gel-filtrazione su P–200 Biorad seguita da cromatografia su CM–50 Sephadex. L'applicazione di 2 gradienti lineari, uno dei quali contiene citrato, a due successivi stadi della cromatografia ha permesso di separare l'attività dell'aconitato–idratasi da quella della isocitrico–deidrogenasi in 2 frazioni omogenee purificate rispettivamente 14 e 30 volte. Sugli enzimi purificati il meccanismo d'inibizione prodotto dall'ossalomato è stato reinvestigato.

Oxalomalate, a tricarboxylic acid synthesized by chemical condensation of oxaloacetate with glyoxylate was found by Ruffo *et al.* (1962–1974) to inhibit at very low concentration either aconitate hydratase (E. C. 4.2.1.3) or isocitrate(NADP)dehydrogenase (E. C. 1.1.42).

In order to compare the mechanism of inhibition on both the enzymes which are known to catalyze fundamental reactions connected with the citric acid cycle it appeared of interest to purify the enzymes from the same mitochondrial preparation.

Combining and modifying the ion exchange chromatography described by MacFarlane *et al.* (1977) with the gel-filtration method of Colman (1968) for the purification of isocitrate(NADP)dehydrogenase and applying two different linear gradients on successive stages of the chromatographic separation, we succeeded in obtaining from an extract of beef heart mitochondria, besides isocitrate-dehydrogenase, another homogeneous fraction containing aconitate-hydratase. In the course of the procedure malic dehydrogenase was also identified, separated and collected as a highly purified fraction.

MATERIAL AND METHODS

Oxoacids, cisaconitate and D-L-isocitrate (type I) were from SIGMA (St. Louis, USA), oxidized and reduced coenzymes were from Boehringer (Menneihm, Germany). P-200 Biorad was supplied by Biorad Laboratoires

(*) Istituto di Chimica Biologica dell'Università di Pavia.
(**) Divisione di Biochimica Istituto dei Tumori di Napoli.
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(Richmond, California, USA), CM-50 Sephadex by Pharmacia Fine Chemicals (Upsala, Swéden), Dodecylsulfate-Na Salt (SDS), Serva F. B. (Heidelberg, Germany) and EDTA and mercaptoethanol FLUKA, A. G. (Buchs S. G. Switzerland). All other analytical grade reagents were from C. Erba (Milano, Italy) or E. Merck (Darmstad, Germay).

Chemical and analytical methods. 4-hydroxy-2-oxoglutarate was prepared by acidification of oxalomalate according to the method Ruffo *et al.* (1967). Ketonic functions were determined by the colorimetric method (Lardy, 1959) and protein concentration at 280 and 260 nm (Kalkar, 1947).

Preparation of the enzymes.

a) Mitochondria: the beef heart free of blood and fat was minced and homogenized using a cold Waring Blandor in 3.6 vols of 0.05 M Na-phosphate buffer, 0.01 M KCl, 0.001 M EDTA pH 6.5. After adjusting the pH to 8.3 with 1-N-NaOH the homogenate was centrifuged at 1.500×g to remove nuclei and cell debris. The supernatant was collected and the precipitate washed twice with the same buffer. The supernatant and the two washings were pooled, filtered thorugh cheese cloth and centrifuged for 35 min. at 15.000×g. The mitochondria were washed twice with 1 mM EDTA pH 7.0 containing 0.1 % mercaptoethanol and suspended in 500 ml of 0.05 M Na-phosphate buffer, 1 mM EDTA, 0.1 % mercaptoethanol pH 7.0. The mitochondrial suspension was homogenized into a stainless VIRTIS homogenizer by 6 runs of 30 m sec each at 80.000 r.p.m. The extract was then centrifuged at 48.000×g for 60 min. All operations were carried out at 4 °C.

b) *Fractionation with* $(NH_4)_2SO_4$. Solid $(NH_4)_2SO_4$ (30 gr/100 ml of extract) was added to the supernatant (ca. 450 ml) taking care to keep the pH between 7 and 7.4. The precipitate was discarded and solid $(NH_4)_2SO_4$ (23 g/100 ml) added to the supernatant to obtain a concentration of 53 %. The precipitate was dissolved in a minimum volume of 0.05 M phosphate buffer pH 6.2, containing 10 % glycerol, 1 mM EDTA, 0.1 % mercaptoethanol (Buffer A).

c) *Gel-Filtration.* The enzyme solution was loaded onto a column (100× 4.8 cm) of P-200 Biorad equilibrated with buffer A and eluted at the rate of 12–15 ml/hour with the same buffer. The fractions between 550 and 700 ml containing the activities of aconitate-hydratase, malic and isocitric-dehydrogenase were pooled and concentrated to 15–19 ml by pressure filtration using a PM-30 membrane in an Amicon apparatus.

d) *Ion exchange chromatography.* The enzyme preparation was loaded onto a CM-50 Sephadex column (32×3 cm) equilibrated with buffer A and the inactive proteins were washed out with this buffer. Elution was carried out by using a linear gradient of 0.03 M $MgSO_4$ and 0.125 M Na_2SO_4 in buffer A. The activity of aconitate hydratase, malic dehydrogenase and isocitrate dehydrogenase was recovered in three fractions between 450 and 900 ml. (See Fig. 1 and Table I).

e) *Rechromatography*. The second fraction containing aconitate hydratase and malic dehydrogenase was concentrated on a PM-30 membrane as before and rechromatographed on a second CM-50 Sephadex column equilibrated with buffer A. Elution was performed using a linear gradient of 0.03 M $MgSO_4$, 0.065 M Na_2SO_4 and 0.03 M citrate in buffer A (Fig. 2).

The third fraction, containing isocitrate and malic dehydrogenase, was rechromatographed on a P-200 Biorad column (90×2 cm) equilibrated with a 0.1 M phosphate buffer containing 1 mM EDTA, 10 % glycerol and 0.8 % mercaptoethanol pH 7.6 (Buffer B) (See Fig. 3 and Table I).

The various purification steps were monitored by spectrophotometric assay and by electrophoresis in 10 % polyacrilamide gel in the presence of 0.1 % sodium dodecylsulphate (SDS) (Weber and Osborn, 1969).

Determination of enzyme activities.

a) Aconitate hydratase: the inactive protein was reactivated by preincubation with Fe^{2+} and reducing agents as follows: 0.2 ml of the enzyme solution was preincubated for 30 min. at 37 °C in 0.5 M Tris–HCl buffer pH 8 containg 50 mM dithioerythrol and 1.2 mM $(NH_4)_2Fe(SO_4)_2$ in the final volume of 0.6 ml. All solutions and water were previously degassed. The activity was determined on 0.1 ml of the incubated mixture at 340 nm according to the method of Siebert (1963) using an ACTA III Beckman Spectrophotometer.

Activity was expressed as micromoles of NADP reduced in 1 minute, measured between 1 and 2 min. from the start of the reaction. For the kinetic experiments the same assays with citrate or cisaconitate were performed on a Beckman spectrophotometer DK–1 A, reducing the final volume to 1 ml. When isocitrate was the substrate, cis-aconitate formation was recorded at 240 nm with a Beckman DK–1 A spectrophotometer according to the method of Racker (1950).

b) Isocitrate dehydrogenase: determined in a silica cuvette of a Beckman DK–1 A spectrophotometer containing 10 mM phosphate buffer pH 7.4, 2 mM D-L-isocitrate, 25 mM $MgCl_2$ and 0.125 mM $NADP^+$ in the final volume of 3 ml. Readings at 340 nm between 0 and 2 min. were taken and the results expressed as micromoles of $NADP^+$ reduced in 1 min.

c) Malate dehydrogenase: determined in a cuvette containing 0.1 ml of 1 M L-malate, 0.1 ml of 12.3 mM NAD and 0.09 M glycine-NaOH buffer pH 10 to a volume of 3 ml. The amount of enzyme was chosen so that the initial increase of O.D. was approsimately 0.05 for 1 min. The results are expressed as micromoles of NAD reduced in 1 min. at 340 nm.

RESULTS AND DISCUSSION

1. *Purification of the enzymes*. The results reported in Fig. 1–3 and Table I show that the technique described permits either aconitate hydratase

or isocitrate dehydrogenase, extracted from the same preparation of mitochondria, to be separated and obtained as homogeneous fractions. Isocitrate dehydrogenase was separated in the course of the first elution from Sephadex CM–50 slightly contaminated by malic dehydrogenase. Aconitate hydratase was eluted from the same column in two peaks, the second one being associated with malic dehydrogenase (Fig. 1). Separation of the two enzymes

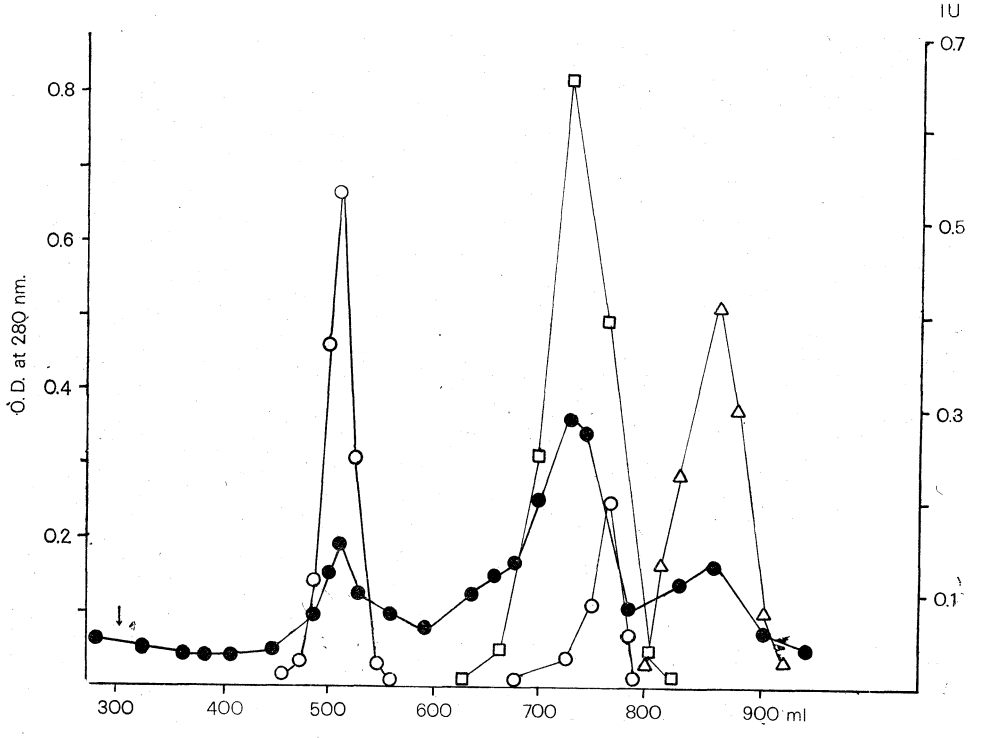

Fig. 1. – Separation of the enzymic activities by the 1st Sephadex column. Composition of the samples reported in the text: left side OD at 280 nm (●); right: I.U. of aconitate hydratase/0,2 ml (○); malic dehydrogenase/0.01 ml (□); isocitric–dehydrogenase/0,01 ml (△). The arrow indicates the application of the gradient.

was obtained when this fraction was rechromatographed on the II CM–50 Sephadex (Fig. 2). When the 1st peak of aconitate hydratase was rechromatographated on CM–50 Sephadex all activity was recovered in the position previously occupied by the 2nd peak, suggesting that the association of aconitate hydratase activity with two different proteins may depend either on aggregation of the enzyme with another protein, or on protein-protein interaction. This problem is still under investigation. With regard to isocitrate dehydrogenase, by repeating the passage on another Biorad column eluted at pH 7.6, an homogeneous fraction free of malic dehydrogenase (see Fig. 3) was collected.

Aconitate hydratase and isocitrate dehydrogenase presented only one band when analyzed by disc-electrophoresis but malic dehydrogenase was still contamined by another small band. With regard to the specific activity and recovery of the enzymes, the data in Table I show satisfactory results which suggest that the method may be extended for separating other soluble enzymes contained in mitochondria.

TABLE I.

Simultaneous separation and purificazion of Aconitate hydratase (ACH), *Isocitrate dehydrogenase* (ICDH) *and Malic dehydrogenase* (MDH) *from hert beef mitochondria.*

(Experimental conditions described in the text).

FRACTIONS	ENZYMES	VOLUMES ml	ACTIVITY I. U.	PROTEINS mg	S. A. I. U./mg
1) Mitochondrial extract	ACH	570	165	1083	0.15
	MDH	570	8242	1083	7.60
	ICDH	570	1649	1083	1.52
2) $(NH_4)_2SO_4$ precipitate	ACH	20	160	408	0.39
	MDH	20	7090	408	17.04
	ICDH	20	1389	408	3.40
3) P-200	ACH	16.5	96	191	0.5
	MDH	16.5	6128	191	32.0
	ICDH	16.5	955	191	5.0
4) CM-50	ACH	6.4	20	11	1.8
	ACH (*)	10.5	25	30	0.8
	MDH (*)	10.5	2887	30	95.0
	ICDH	12.5	482	15	32.0
5) II CM-50	ACH	7.7	8	3.6	2.2
	MDH	3.2	1700	7.2	238
6) II P-200	ICDH	10.5	456	8.8	52

(*) Both contained in the II fraction between 600 and 800 ml (see Fig. 1).

2. *Inhibition by oxalomalate.* Using the purified preparations the mechanism of inhibition by oxalomalate and its decarboxylation product 2-hydroxy-4-oxoglutarate was reinvestigated in order to compare the sensitivity of the pure enzymes to the inhibitors. The main results confirmed the competitive inhibition of both the enzymes (see Fig. 4) provided that the inhibitors were added to the samples together with the substrates. Hydroxyoxoglutarate was found to be 10 times less efficient than oxalomalate.

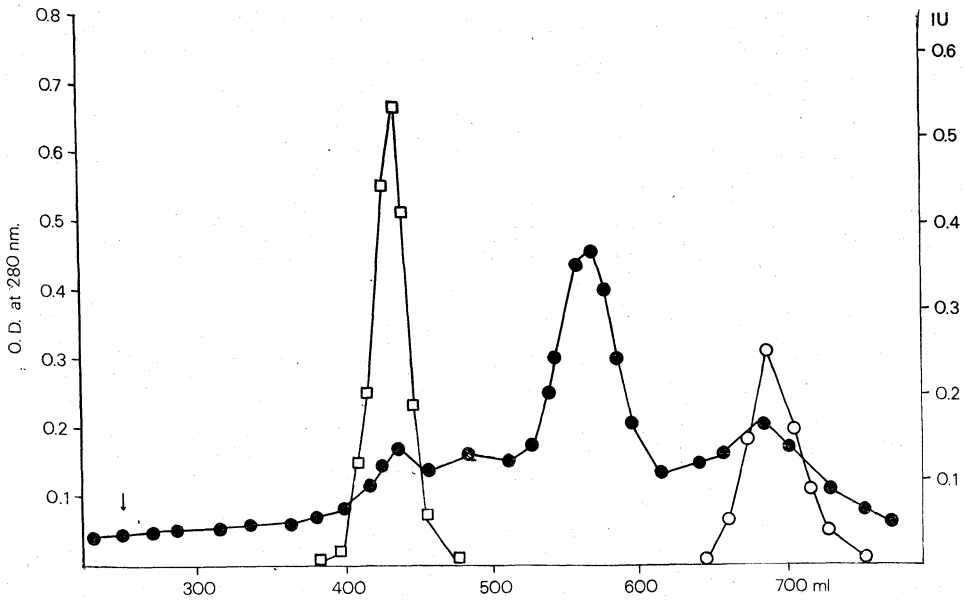

Fig. 2. - Purification by rechromatography. Experimental conditions reported in the text: left side OD 280 nm (●); right I.U. of aconitate hydratase/0.2 ml (○); malic dehydrogenase/ 0.01 ml (□). The arrow indicates the application of the gradient

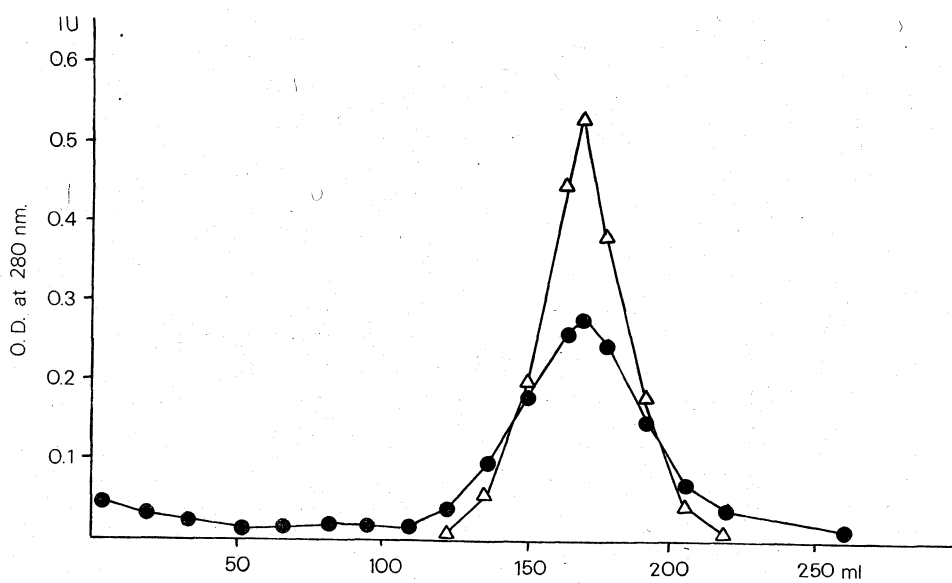

Fig. 3. - Isolation of isocitrate-dehydrogenase. Experimental conditions reported in the text. On the ordinate either the OD (●) or the I.U. of isocitrate-dehydrogenase contained in 0.005 ml (△). On the abscissa the ml of eluent

We determined the Ki for oxalomalate using citrate, cis-aconitate and isocitrate as substrates. The results with aconitate hydratase were 1.2×10^{-5} M for citrate and 8.09×10^{-6} M for cis-aconitate. With isocitrate as substrate the Ki was 2×10^{-5} M. Similar experiments were repeated with isocitrate dehydrogenase and the Ki for oxalomalate was 1.4×10^{-5} M.

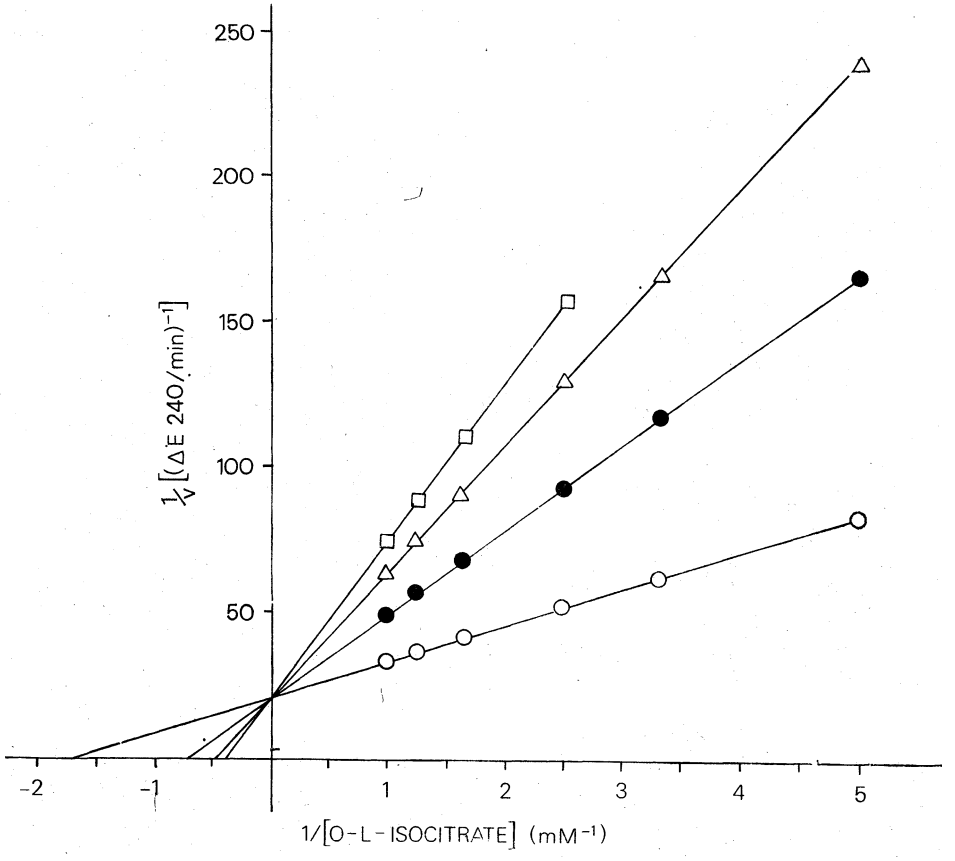

Fig. 4. – Competitive inhibition of aconitate hydratase by oxalomalate. Each incubation mixture prepared directly in the quarz cuvettes of a Beckman Spectrophotometer contained D-L-isocitrate as indicated and 0.05 M phosphate buffer pH 7.4; The reaction was started by adding 0.005 ml of reactivated enzyme (see text) corresponding to about 0.005 I.U. (○) Control; oxalomalate: (●) 0.33 mM; (△) 0.48 mM; (□) 0.66 mM. The ΔE_{240} per min. was taken as a measure of the enzyme activity.

Moreover, in other experiments both enzymes were preincubated in order to investigate the time dependence of the inhibitory process. Preincubation even for 2 min. of both enzymes with a low concentration (0.1 mM) of oxalomalate produced irreversible inhibition not modified by the addition of an excess of substrates, suggesting an apparent time dependent inactivation of both enzymes.

Since recent results (Adinolfi, Menna and Ruffo, 1980) have shown that either oxalomalate or hydroxyoxoglutarate may be reduced by isocitrate dehydrogenase and NADPH to compounds inactive on the enzymes, it seems likely that these reactions may protect the whole cycle by inhibitory control. The conversion of oxalomalate into an inactive compound may also be the cause of the reversibility of the inhibition of aconitate hydratase observed *in vivo* by Romano *et al.* (1960) following the injection in rats of an equimolar solution of glyoxylate and oxaloacetate, which are the precursors of oxalomalate. Since this powerful inhibitor may be formed and enzymatically converted into an inactive product under physiological conditions of temperature and pH, we conclude that the mechanism of inhibition described probably plays an important role in the regulation of the tricarboxylic acid cycle.

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