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The sodium ion pumping oxaloacetate decarboxylase of *Klebsiella pneumoniae*

Metal ion content, inhibitors and proteolytic degradation studies

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Oxaloacetate decarboxylase of *Klebsiella pneumoniae* was shown to contain between 0.6 and 1.0 mol zinc per mol enzyme in different preparations. The decarboxylase activity was completely abolished after 15 min incubation with 1 mM $Hg(NO_3)_2$ in phosphate buffer, while the activity decreased only 20% if the incubation was performed in MES/Tris buffer. Treatment of the isolated subunits with $Hg(NO_3)_2$ indicated that the binding site for Hg^{2+} ions is on the α subunit. Other inhibitors of the decarboxylase are KSCN and diethylstilbestrol. Inactivation of the enzyme with 2% 1-butanol was significantly reduced by 100 mM NaCl. Sodium ions also protected the isolated β+γ subunits from a digestion with trypsin.

Oxaloacetate decarboxylase; Zinc content; Enzyme inhibition; Protection of proteolysis by Na^+ ions

1. INTRODUCTION

Oxaloacetate decarboxylase, methylmalonyl-CoA decarboxylase and glutaconyl-CoA decarboxylase belong to a family of enzymes that catalyze the electrogenic transport of Na^+ ions upon decarboxylation of each specific substrate (for review see [1,2]). The subunit composition and the catalytic mechanism of all Na^+-translocating decarboxylases appear to be very similar [3–5]. The first step is the transfer of the carboxyl group from the substrate to the prosthetic biotin group (Eqn. 1). This reaction is catalyzed by a peripheral membrane-bound subunit (α) which has a molecular weight of about 65,000 [3,5,6]. The biotin resides either on the C-terminal domain of the α subunit (oxaloacetate decarboxylase [3]) or on a separate biotin carrier protein subunit with M_r 18,000–24,000 (methylmalonyl-CoA decarboxylase and glutaconyl-CoA decarboxylase [4,5]). The carboxyltransferase reaction which is completely independent of the presence of Na^+ ions is succeeded by the Na^+-dependent decarboxylation of the enzyme-bound carboxybiotin that is coupled to Na^+ translocation (Eqn. 2). This reaction is catalyzed by the more firmly membrane-bound subunits β and γ in oxaloacetate decarboxylase or β and δ in methylmalonyl-CoA decarboxylase which have molecular weights of about 40,000 (β) and 9,000 (γ) and 14,000 (δ) [3–6].

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$$R\text{-}COO^- + E\text{-biotin} \rightleftharpoons RH + E\text{-biotin-}CO_2^- \quad \text{(Eqn. 1)}$$

$$E\text{-biotin-}CO_2^- + H^+ + 2Na^+_{in} \rightleftharpoons E\text{-biotin} + CO_2 + 2NA^+_{out} \quad \text{(Eqn. 2)}$$

More recently, the oxaloacetate decarboxylase of *Klebsiella pneumoniae* has been cloned and sequenced. The N-terminal part of the sequence of the α subunit is highly homologous to the 5S subunit of transcarboxylase from *Propionibacterium shermanii* which catalyzes the same carboxyltransfer reaction (Eqn. 1) [7]. The C-terminal part of the α subunit sequence resembles the sequences of biotin carboxyl carrier protein subunits/domains of several biotin enzymes [7,8]. These sequences are one precondition for defining the catalytic sites of these enzymes, but additional knowledge of structure and catalytic properties are also required. Further studies on the structure and catalytic mechanism of oxaloacetate decarboxylase were therefore performed and are described below.

2. EXPERIMENTAL

2.1. *Materials*

Oxaloacetate decarboxylase of *Klebsiella pneumoniae* was prepared by affinity chromatography of a solubilized membrane extract on monomeric avidin-Sepharose [9].

The purified enzyme was dissociated by freezing and thawing in the presence of $LiClO_4$ and the biotin containing α subunit was separated from (β+γ) by chromatography on monomeric avidin-Sepharose as described [10].

2.2. *Determination of tightly-bound metal ions in oxaloacetate decarboxylase*

Purified solutions of oxaloacetate decarboxylase containing 4–8 mg

protein/ml were dialyzed overnight against 10 mM Tris-HCl buffer, pH 7.4. Samples of the dialyzed enzyme were used directly or after dilution with deionized water for metal ion analyses. The content of metal ions was determined by atomic absorption spectroscopy using a Shimadzu AA 646 atomic absorption/flame emission spectrophotometer. Each element was determined at three different dilutions under conditions of optimal sensitivity as described by the manufacturer. The dialysis buffer was used as a blank. The calibration curves were linear at least up to 20 μM of the metal ions analyzed and yielded at 1 μM concentration for Co^{2+} $\Delta E = 0.003$, for Zn^{2+} $\Delta E = 0.02$ and for Mn^{2+} $\Delta E = 0.005$.

3. RESULTS AND DISCUSSION

3.1. *Content of tightly bound Zn^{2+} in oxaloacetate decarboxylase*

Oxaloacetate decarboxylase, pyruvate carboxylase and transcarboxylase are enzyme complexes that share a common reaction, i.e. the transfer of the carboxyl group from oxaloacetate to enzyme-bound biotin (Eqn. 1). The sequences of the three different carboxyltransferase subunits are highly conserved suggesting that they are descendants from a common ancestor and probably react by a common mechanism [7,8]. In accord with this notion is the same stereochemical course by retention of configuration in all three cases [1,11]. Pyruvate carboxylase contains tightly-bound manganese in the active center of the carboxyltransferase. The participation of this metal ion in the carboxyltransfer reaction has been directly demonstrated by NMR spectroscopic techniques [12]. The 5S subunit of the transcarboxylase from *Propionibacterium shermanii* contains tightly-bound cobalt and zinc with an apparently analogous function as the manganese in pyruvate carboxylase [13].

It was of interest, therefore, to determine, whether oxaloacetate decarboxylase also contained tightly-bound metal ion(s). Dialyzed samples of the purified enzyme were analyzed for manganese, cobalt and zinc by atomic absorption spectroscopy. The enzyme contained neither manganese nor cobalt but zinc ranging between 0.61 and 0.99 mol per mol of enzyme in the different preparations analyzed (Table I). These data suggest the participation of Zn^{2+} in the carboxyltransferase reaction of oxaloacetate decarboxylase, analogous to the function of Mn^{2+} or Co^{2+} and Zn^{2+} in pyruvate carboxylase or transcarboxylase, respectively [12,13].

Table I

Metal ion content of oxaloacetate decarboxylase. Different preparations of oxaloacetate decarboxylase were dialyzed and subjected to the analyses of Co^{2+}, Zn^{2+} and Mn^{2+} by atomic absorption spectroscopy as described in section 2. Mean values obtained with three different dilutions are given. The molar content of oxaloacetate decarboxylase was calculated on the basis 1 nmol = 0.118 mg protein.

Oxaloacetate decarboxylase		Co^{2+}	Mn^{2+}	Zn^{2+}	Zn^{2+}/ enzyme
Preparation	(μM)		(μM)		(mol/mol)
1	3.4	0	0	3.0	0.88
2	2.6	0	0	2.3	0.88
3	3.3	0	0	2.0	0.61
4	14.0	0	0	12.0	0.86
5	8.5	0	0	8.4	0.99

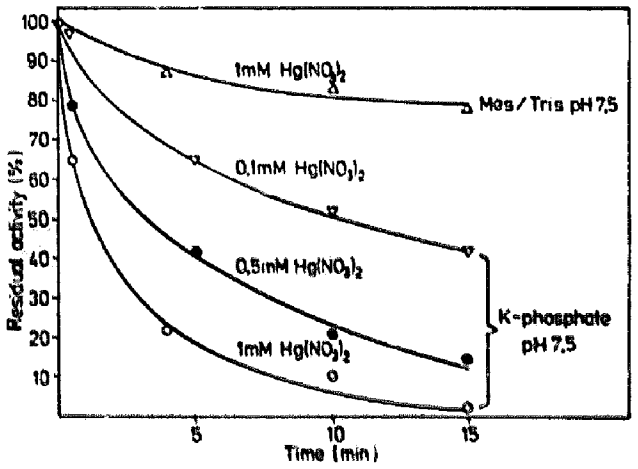

Fig. 1. Time course of the inhibition of oxaloacetate decarboxylase with $Hg(NO_3)_2$. The enzyme was incubated at 25°C in 200 mM MES/Tris buffer, pH 7.5 or 100 mM potassium phosphate buffer, pH 7.5 with the concentrations of $Hg(NO_3)_2$ indicated. At the times indicated, samples were diluted 200-fold into cuvettes containing the reagents for determining the oxaloacetate decarboxylase activity.

3.2. *Inhibitors of oxaloacetate decarboxylase*

Several compounds known to react with SH-groups of enzymes were tested as possible inhibitors of oxaloacetate decarboxylase. No significant inhibition was found with iodoacetate, iodoacetamide, 5,5'-dithio-bis-(2-nitrobenzoate), 2,2'-dithiodipyridine and *N*-ethylmaleinimide. Mercurials were on the other hand effective inhibitors of the decarboxylase. As an example, the time course of inhibition of oxaloacetate decarboxylase with different concentrations of $Hg(NO_3)_2$ is shown in Fig.

Table II

Effect of $Hg(NO_3)_2$ on the catalytic activity of oxaloacetate decarboxylase subunits. The isolated α or the $(\beta+\gamma)$ subunits were incubated in a total volume of 0.16 ml 0.1 M potassium phosphate buffer, pH 7.5 with 1.2 mM $Hg(NO_3)_2$ for 15 min at 25°C. Controls without $Hg(NO_3)_2$ treatment were run in parallel. All samples were dialyzed for 3 h against 50 mM Tris-HCl buffer, pH 7.5. The enzyme complex was then reconstituted from the subunit fractions by 30 min incubation at 25°C. The oxaloacetate decarboxylase activities were subsequently determined and are given as percentage of the reconstituted enzyme from subunits not treated with $Hg(NO_3)_2$.

Subunit combinations	Oxaloacetate decarboxylase (%)
α (untreated) + $(\beta+\gamma)$ (untreated)	100
α (Hg^{2+}) + $(\beta+\gamma)$ (Hg^{2+})	4
α (Hg^{2+}) + $(\beta+\gamma)$ untreated	6
α (untreated) + $(\beta+\gamma)$ (Hg^{2+})	68

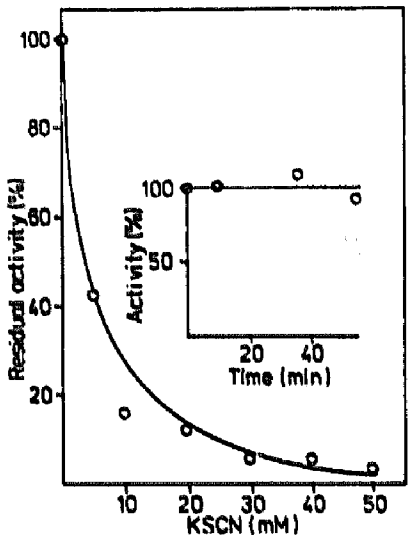

Fig. 2. Inhibition of oxaloacetate decarboxylase by KSCN. The activities were determined in 100 mM potassium phosphate buffer, pH 7.5 by the direct spectrophotometric assay at 265 nm [9] in presence of the KSCN concentrations indicated. The inset shows the reversibility of the inhibition with KSCN. The enzyme was incubated for the times indicated with 6.6 mM KSCN and the oxaloacetate decarboxylase activities were determined after 67-fold dilution into assay mixture not containing KSCN.

1. A concentration of 1 mM $Hg(NO_3)_2$ was sufficient to completely inactivate the enzyme within 15 min, if the incubation was performed in phosphate buffer, pH 7.5. Surprisingly, the activity decreased only 20% in MES/Tris buffer under otherwise identical conditions. A conformation of the enzyme may therefore be stabilized by MES/Tris buffer, by which specific SH groups are protected from a reaction with Hg^{2+}. The reaction of Hg^{2+} with SH groups was indicated by partial reactivation of the $Hg(NO_3)_2$-inhibited enzyme after incubation with dithioerythritol.

The presence of 50 mM pyruvate protected the decarboxylase from inhibition by $Hg(NO_3)_2$ in phosphate buffer to the same extend as MES/Tris buffer did. The inhibition was not effected by 10 mM NaCl. The target for the reaction with Hg^{2+} ions could therefore be on the carboxyltransferase subunit. This was shown by an incubation of the isolated α subunit or the $(\beta+\gamma)$ subunits with $Hg(NO_3)_2$ followed by dialysis to remove residual $Hg(NO_3)_2$ and reconstitution of the enzyme complex. The results shown in Table II indicate loss of reconstitutable decarboxylase activity after treatment of the α subunit with $Hg(NO_3)_2$ but only partial loss of activity if the $(\beta+\gamma)$ subunits were incubated with the mercury salt.

The results of Fig. 2 show the decrease of oxaloacetate decarboxylase activity at increasing concentrations of KSCN, reaching 95% inhibition in presence of 50 mM of this compound. This inhibition was completely reversible, as shown by 100% recovery of catalytic activity upon dilution following incubation of the enzyme with 6.6 mM KSCN up to 55 min. Diethylstilbestrol was found to be another inhibitor of the decarboxylase (Fig. 3). The inhibition is characterized by

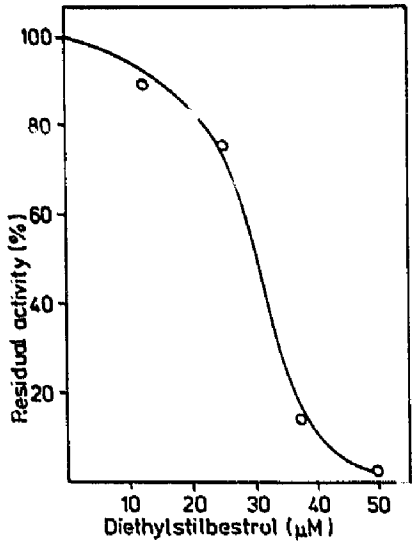

Fig. 3. Effect of diethylstilbestrol concentration on oxaloacetate decarboxylase activity. The coupled spectrophotometric assay with lactate dehydrogenase was used with 100 mM Tris-HCl buffer, pH 7.5 [9]. The cuvettes contained the diethylstilbestrol concentrations indicated.

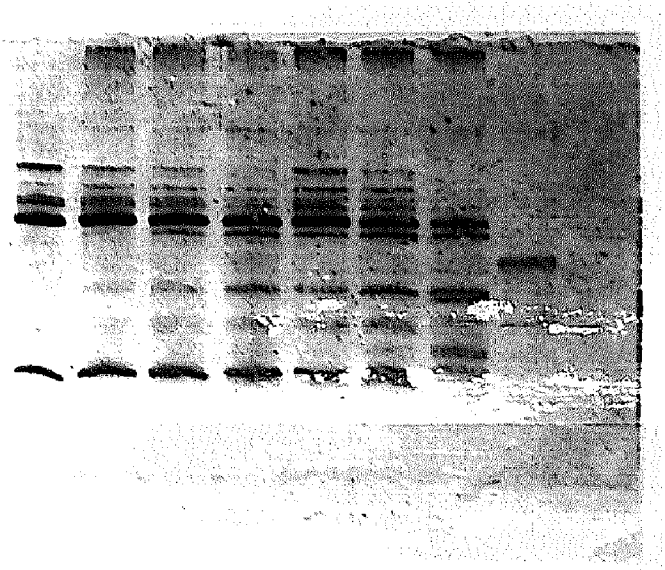

Fig. 4. Proteolytic degradation of the β and γ subunits of oxaloacetate decarboxylase with trypsin and effect of Na^+ ions on this proteolysis. The incubation mixtures contained in 0.25 ml at 25°C: (A) 50 mM MES/Tris buffer, pH 6.0, the isolated $(\beta+\gamma)$ subunits (50 μg protein) and 0.25 μg trypsin. A parallel incubation mixture (B) contained 100 mM NaCl in addition. Samples (50 μl) were transferred after 1, 3 and 10 min incubation into 50 μl 10% formic acid. The solvent was removed in vacuo, the residues dissolved in 50 μl sample buffer and after heating to 100°C for 3 min 5 μl aliquots were subjected to SDS gel electrophoresis. The samples applied are from left to right: the $(\beta+\gamma)$ subunits without trypsin, tryptic digestion mixture A after 1 min, after 3 min, after 10 min, tryptic digestion mixture B after 1 min, after 3 min, after 10 min, trypsin.

an S-shaped profile with an inflection point at around 30 μM diethylstilbestrol and a decrease to less than 5% activity in presence of 50 μM of this compound.

3.3. *Effect of Na^+ on the inactivation and digestion of decarboxylase subunits*

The oxaloacetate decarboxylase activity was destroyed in the presence of alcohols with increasing efficiency in going from ethanol over propanol to butanol. The enzymic activity decreased 85% during a 10 min incubation with 2% *n*-butanol. The additional presence of 100 mM NaCl largely protected the enzyme from this inactivation. The results are analogous to similar observations with glutaconyl-CoA decarboxylase [5] and may suggest that in the presence of Na^+ these decarboxylases assume a more compact conformation. This notion is also in accord with results from proteolytic degradation which indicated a specific protection of the β-chains of all three different decarboxylases by Na^+ ions [3,5,6]. To determine whether this protective effect of Na^+ ions required the structurally intact enzyme complexes, or whether a similar interaction with Na^+ was also possible with the isolated ($\beta+\gamma$) subunits, we studied the digestion of these subunits from oxaloacetate decarboxylase by trypsin. The results shown in Fig. 4 indicate that in the absence of Na^+ ions the β subunit is degraded to several distinct fragments. The γ subunit appears to be digested through a fragment of intermediate size to small fragments that are not detectable on the SDS gel. In the presence of Na^+ ions, the β subunit was clearly protected from the tryptic digestion, and also the γ subunit was not significantly degraded in the absence of the alkali ion. These results could indicate that the Na^+ binding site is contributed by both β and γ subunits. It is also evident that the α subunit is not required for the binding of Na^+ by ($\beta+\gamma$) that results in a conformational change of these subunits. These observations are in accord with recent results of Na^+ translocation by the isolated ($\beta+\gamma$) subunits. The specific activity for Na^+ translocation by ($\beta+\gamma$) in response to $\Delta\tilde{\mu}Na^+$ was about the same as that for active Na^+ transport by the oxaloacetate decarboxylase complex (manuscript submitted).

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