MOLECULAR STRUCTURE OF MALATE SYNTHASE AND STRUCTURAL CHANGES UPON LIGAND BINDING TO THE ENZYME

G. Schmid, H. Durchschlag, G. Biedermann, H. Eggerer and R. Jaenicke

Biochemie I/II, Fachbereich Biologie, Universität Regensburg, Germany

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SUMMARY. Malate synthase has a molecular weight of about 170 000 as shown by ultracentrifugation, sucrose gradient centrifugation, and thin layer gel-chromatography. High dilution, extremes of pH, succinylation, and treatment with sodium dodecylsulfate suggest the enzyme to be a tetramer. The CD spectrum is typical for a globular protein with moderate helical content (~30 %), and shows anomalous Cotton effects at 250-290 nm. Binding of substrates (acetyl-CoA, glyoxylate) or the substrate analog pyruvate causes slight conformational changes which are reflected in alterations of the CD bands in the range of aromatic absorption; binding of Mg^{2+} causes no structural effects, suggesting the metal ion to be involved in enzymatic catalysis rather than structural alterations.

INTRODUCTION. Malate synthase (EC 4.1.3.2) is a shunt-enzyme in the anaplerotic glyoxylic acid cycle (1), and catalyzes the synthesis of 2 S-malate from acetyl-CoA and glyoxylate (2):

$$HOOC\text{-}CHO + CH_3COSCoA + H_2O \rightleftharpoons HOOC\text{-}CH(OH)CH_2\text{-}COOH + CoA\text{-}SH \quad [1]$$

The enzyme requires Mg^{2+}; it enolizes acetyl-CoA and hydrolyzes malyl-CoA, the enzymatic enolization depending strictly on magnesium. In the absence of the substrate glyoxylate the enolization proceeds a thousand times slower than in the case of reaction [1]. In the presence of the substrate-analog pyruvate the enolization is stimulated to reach nearly the velocity of malate synthesis (3). On the basis of this observation the action of Mg^{2+} and pyruvate were considered to cooperate in an acid-base catalysis. On the other hand, these ligands may induce conformational transitions, this way unmasking a catalytic group of the enzyme.

In order to determine whether conformational changes are involved in the mechanism of the enzyme, both the molecular structure of MS and the influence of Mg^{2+}, glyoxylate, pyruvate, and acetyl-CoA on the structure of the native enzyme were investigated.

Abbreviations: MS, malate synthase (EC 4.1.3.2); CD, circular dichroism

No alterations of the particle weight were observed upon adding the cofactor or the substrates (3); on the other hand high dilution of the enzyme leads to partial dissociation which seems to be reversed by excess of substrate (4). As shown by CD spectroscopy, binding of the ligands to MS in the presence and absence of Mg^{2+} leads to slight conformational changes which are reflected by spectral changes in the range of absorption of the aromatic chromophores. Mg^{2+} alone does not cause significant effects.

MATERIALS AND METHODS. MS from baker's yeast was purified according to Biedermann (5); the optical test was performed using the method of Eggerer and Klette (3); the specific activity of the electrophoretically pure enzyme was 22-25 IU/mg. The concentration of the enzyme was determined by the biuret method. Mg^{2+}-free enzyme was prepared by incubating MS with 0.01 M EDTA for 20 minutes at 5°C in 0.05 M phosphate buffer, pH 7.8 (5); after removal of Mg·EDTA by rinsing MS with phosphate buffer in an Amicon diaflo apparatus the enzyme was completely inactive. After addition of 10 mM $MgCl_2$ 100 % activity was restored immediately. Acetyl-CoA was prepared by the method of Simon and Shemin (6); for further purification the method of Hurlbert (7) was used. Pyruvate was purified according to v. Korff (8).
CD spectra were measured in a Jouan-Roussel Dichrographe II (scale expansion $\Delta E = 2 \cdot 10^{-6}$/mm) in thermostated quartz cuvettes; the pathlength of the cuvettes was 0.1-100 mm.
In order to reduce systematic errors CD spectra were run in cuvettes in a "tandem" arrangement. For this purpose different combinations of the enzyme solution with and without the ligands were compared. As an example, the difference of the spectra of enzyme + buffer and substrate + buffer on one hand, and enzyme + substrate + buffer and buffer alone on the other hand provides the intrinsic spectral effect of the formation of the enzyme-substrate complex. In this way the complexes of MS with Mg^{2+} and the substrates were investigated in separate scans.
Ultracentrifugation analysis made use of sedimentation velocity and sedimentation equilibrium runs in a Beckman ultracentrifuge (Model E) equipped with schlieren optics and a high sensitivity UV scanner system. 12 mm and 30 mm double sector cells were used in the wavelength range between 230 and 280 nm. Gradient centrifugation was performed according to Martin and Ames (9) in a Beckman ultracentrifuge (SpincoL), thin layer gel-chromatography according to Determann (10).

RESULTS. In order to investigate the structural alterations upon ligand binding the molecular parameters of MS in the native state had to be analyzed as a reference. The characterization refers to the determination of both particle weight and conformational parameters.

Molecular weight. The molecular weight of MS in the presence of magnesium was determined by gradient centrifugation using aldolase and catalase as

references: M = 161 000 ± 12 000. Thin layer gel-chromatography with cytochrome c as internal standard yields M ~ 170 000 (5). High speed sedimentation equilibrium experiments (11) lead to M_w = 169 000 ± 7 000 (5 mM Tris buffer/1 mM $MgCl_2$, pH 8.0, 0.1<c< 1.5 mg/ml). Calculations from sedimentation / diffusion based on sedimentation velocity/synthetic boundary runs confirm this result :

$s^o_{20,w}$ = 8.25 ± 0.10 S ; $D^o_{20,w}$ = 4.5 ± 0.3 F; $M_{s,D}$ = 170 000 ± 10 000.
The partial specific volume was taken to be $\bar{v}$ = 0.735 $cm^3 \cdot g^{-1}$, as calculated from the amino acid composition (Table 1) using the volume

TABLE 1. Amino acid composition of malate synthase

Amino acid	Residues per M = 170 000	Amino acid	Residues per M = 170 000
Asp	170 ± 5	Met	36 ± 2
Thr	90 ± 5	Ile	81 ± 8
Ser	71 ± 6	Leu	124 ± 4
Glu	125 ± 3	Tyr	44 ± 2
Pro	80 ± 4	Phe	52 ± 2
Gly	82 ± 4	Trp	13[a]
Ala	95 ± 3	His	28 ± 3
Val	82 ± 8	Lys	84 ± 5
Cys	not determined	Arg	57 ± 3

a estimated from A_{280} according to Wetlaufer (12)

fractions of the amino acid residues in proteins (13).

Subunits. Treatment of MS with 1 % sodium dodecyl sulfate (14) in the absence or presence of 8 M urea and analysis of the products by polyacrylamide gel-electrophoresis reveal a single band of M = 70 000 ± 7 000 indicating dissociation to dimers under these conditions (5). Preliminary succinylation experiments produced a 1:1 mixture of two components of M ~ 78 000 and ~ 42 000, as shown by sedimentation velocity runs in the

ultracentrifuge (5). This result could arise from incomplete succinylation and may indicate the presence of 4 subunits in the native enzyme.
At low concentrations of the enzyme (c 0.1 mg/ml) dissociation of the enzyme is observed which is a accompanied by partial deactivation. At extremes of pH, and after incubation in 67 % acetic acid the enzyme is split into subunits of approximately one quarter the size of the native molecule (Table 2).

TABLE 2. Sedimentation analysis of malate synthase

Buffer	pH	T°C	c(mg/ml)	specific activity (%)	$s^{c}_{20,solv}$(S)
5 mM Tris/1 mM $MgCl_2$	8.0	3	1.0	100	8.25 ± 0.10
50 mM Phosphate	7.8	3	0.003	53	4.2 ± 0.2
0.2 M glycine/NaOH	11.0	20	0.5	0	3.6
0.2 M glycine/NaOH	12.3	2	2.0	0	1.5 - 3.6
0.2 M glycine/HCl[a]	3.0	4	1.0	0	4 - 6

a after treatment with 67 % acetic acid at 2° C and subsequent dialysis

The UV absorption spectrum of MS does not show significant anomalies at 250-300 nm. On the other hand the CD spectrum is characterized by anomalous Cotton effects in the range of aromatic absorption (Fig. 1a); in the far UV region the typical CD spectrum of a globular protein with moderate helical content (≈30%) is observed (Fig. 1b). At low concentrations of the enzyme a decrease of the characteristic CD bands at 208 and 220 nm is detectable which may be correlated to the concentration-dependent dissociation and deactivation (cf. Table 2).

Conformational Changes. Starting from unliganded Mg^{2+}-free MS the transition to the fully liganded enzyme may be investigated in various steps by varying the number of reactants and the sequence in which they are mixed on forming the quaternary complex.
Basically, no significant changes in the secondary structure of MS could be

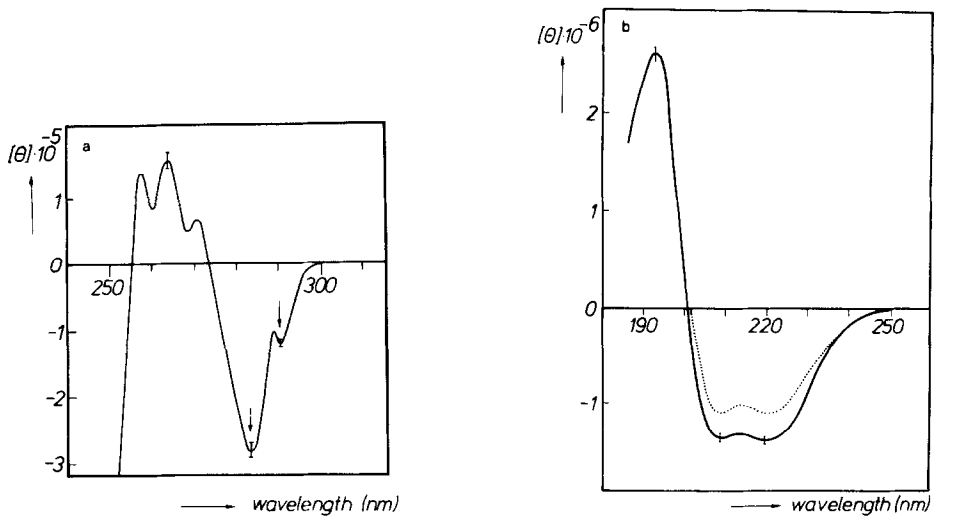

Fig. 1. CD spectrum of malate synthase. 5 mM phosphate buffer pH 7.8, 5°C

a. Range of aromatic absorption, 250-300 nm, c = 1.8 mg/ml, d = 10mm. Tryptophan bands are marked by arrows. Spectra with and without 1 mM $MgCl_2$ are indistinguishable. [Θ] in degrees · cm^2 · mol^{-1}.

b. Far UV range, 185-250 nm.
— c = 2.5 mg/ml, d = 0.1 mm and c = 0.5 mg/ml, d = 0.5 mm;
····· c = 0.0025 mg/ml, d = 100 mm.
Addition of $MgCl_2$, glyoxylate, pyruvate, and acetyl-CoA does not cause significant alterations of the spectrum in the far UV.

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detected upon adding Mg^{2+} or the substrates to the enzyme; this is clearly indicated by the fact that the enzyme in all combinations with its ligands shows the unchanged far UV circular dichroism spectrum characteristic for the native apoenzyme (Fig. 1b). This statement holds within a range of error of ≦ 5 %, which is caused by the unfavourable signal/noise ratio resulting from the high absorption of the quaternary complex (MS· Mg^{2+}· acetyl-CoA· pyruvate).

In the near UV (250-300 nm) addition of Mg^{2+} leads to an unchanged CD spectrum (Fig. 1a). Acetyl-CoA in the presence and absence of Mg^{2+} causes changes in the aromatic CD bands. Similar bands are observed upon glyoxylate and pyruvate addition in the presence of Mg^{2+}; the change of conformation does not take place in the absence of Mg^{2+}. The respective spectra are shown in Fig. 2. Regarding the CD spectrum of the quaternary

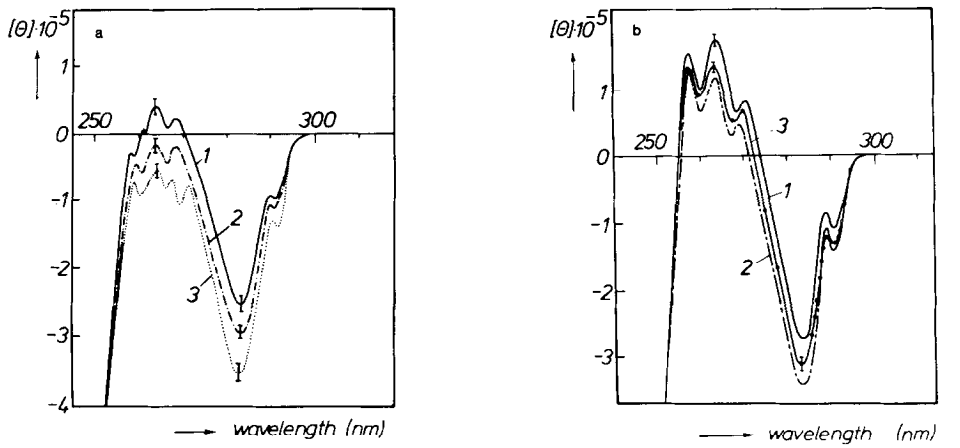

Fig. 2. CD spectra of various complexes of malate synthase (MS) with its substrates, 5°C, c = 1.8 mg/ml, d = 10 mm; $MgCl_2$: 10 mM, pyruvate: 3 mM; glyoxylate: 3 mM; acetyl-CoA: 0.1 mM (cuvette 1/ cuvette 2 in tandem arrangement; buffer in all combinations 5 mM phosphate buffer, pH 7.8).

a. Addition of acetyl-CoA and pyruvate
   1, MS/acetyl-CoA;
   2, MS+acetyl-CoA/buffer = MS+acetyl-CoA+$MgCl_2$/buffer = MS+acetyl-CoA+pyruvate /buffer;
   3, MS+acetyl-CoA+pyruvate+$MgCl_2$/buffer.

b. Addition of glyoxylate and pyruvate
   1, MS/glyoxylate = MS/pyruvate = MS+glyoxylate/buffer = MS+pyruvate/buffer;
   2, MS+$MgCl_2$+glyoxylate/buffer;
   3, MS+$MgCl_2$+pyruvate/buffer.

complex all spectral changes during the single binding steps turn out to be strictly additive and independent of the sequence of complex formation.

DISCUSSION. The determination of the particle weight of malate synthase under various conditions of the medium suggests the native enzyme to be a tetramer. The question whether the subunits are identical or not, cannot be answered so far, neither from ultracentrifugation and amino acid analysis nor on the basis of binding studies with glyoxylate and acetyl-CoA (5). At sufficiently high protein concentrations the enzyme is homogeneous regarding its particle size. Below ~0.1 mg/ml concentration dependent dissociation occurs which is accompanied by partial deactivation. Excess of glyoxylate seems to reestablish the native quaternary structure (4).

As shown by the unchanged sedimentation coefficient (3) and far UV CD spectrum, neither Mg^{2+} nor the substrates cause significant changes of the gross structure of the native apoenzyme. On the other hand, the changes in ellipticity in the aromatic range clearly prove the binding of the substrates to cause slight conformational changes, while Mg^{2+} alone apparently has no structural effect.

From the fine structure of the CD spectrum at 250-300 nm the conclusion may be drawn that aromatic chromophores (phe, tyr, trp) are immobilized in fixed positions in the molecule of the enzyme; the negative bands at 288 and 291 nm may be correlated to a coupling of trp with other aromatic residues (15). As illustrated in Fig. 2 binding of the ligands to the enzyme affects all chromophores responsible for the anomalous bands. From this direct chromophore-chromophore interactions of the substrates with one specific type of aromatic residue in the active center of the enzyme may be excluded as ultimate reason for the observed spectral effects. Furthermore the CD difference spectrum (Fig. 3) clearly indicates that the ellipticity bands

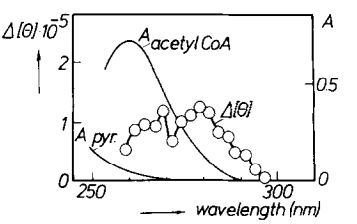

Fig. 3. UV absorption spectra of acetyl-CoA and pyruvate and CD difference spectrum upon ligand binding to malate synthase
——A: UV absorption: 0.1 mM acetyl-CoA, 2.0 mM pyruvate in phosphate buffer; d = 10 mm, 5°C.
o—o : Δ[Θ]: Difference derived from smoothed tandem spectra (MS/acetyl-CoA+pyruvate+buffer) minus (MS·acetyl-CoA·pyruvate/buffer); data from Fig. 2.

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do not originate from extrinsic Cotton effects: If the bands were induced by asymmetric binding of the substrates to the enzyme they should coincide with the absorption maxima of the ligands. Since this is not the case, the spectral changes may be correlated unequivocally to conformational changes of the enzyme.

Taking the changes in ellipticity as a criterion of ligand binding we may conclude that pyruvate and glyoxylate are not bound to the enzyme unless Mg^{2+} is present. On the other hand, binding of acetyl-CoA does take place in the absence of Mg^{2+}. These conclusions are in agreement with the results of binding studies using $[^{14}C]$ acetyl-CoA and $[^{14}C]$ glyoxylate which conclusively demonstrate the aforementioned observations (5). The enolization of acetyl-CoA is catalyzed by Mg^{2+}-MS but not by the Mg^{2+}-free enzyme (3). The metal ion is required for the binding of glyoxylate or pyruvate to the enzyme without by itself changing the enzyme structure on binding. These facts indicate a direct role of the metal ion in the enzyme catalysis (3). The change of conformation found to occur in the Mg^{2+}-dependent binding of glyoxylate or pyruvate however may indicate the unmasking of a catalytic group of the enzyme and therefore give the metal ion an indirect role in the chemistry of this enzyme catalysis.

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