ULTRACENTRIFUGAL AND SPECTROSCOPIC INVESTIGATIONS ON MALATE SYNTHASE FROM BAKER'S YEAST

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1. Introduction

Malate synthase (EC 4.1.3.2) a shunt-enzyme in the anaplerotic glyoxylic acid cycle [1] catalyzes the condensation of acetyl-CoA with glyoxylate to form malate [2]. The enzyme requires Mg^{2+} for activity [3]. It is specific for the substrate glyoxylate and does not show catalytic activity in the presence of the substrate analogues pyruvate, oxaloacetate, α-ketoglutarate etc. [3].

Some enzymological [3–5] and physico–chemical [3,5–8] investigations on the yeast enzyme have been performed earlier. As shown by previous CD-measurements [6] the binding of substrates is accompanied by structural alterations of the enzyme, while binding of Mg^{2+} does not cause significant structural effects.

In the present experiments sedimentation analysis, as well as CD- and fluorescence-spectroscopy were applied to measure structural changes of the enzyme under the same conditions regarding buffer, temperature and specific activity as used for small-angle X-ray experiments [9]. The enzyme shows a concentration-dependent decrease of specific activity and dissociation upon high dilution. Binding of the substrates to the apoenzyme, at concentrations where no dissociation occurs, causes small but significant conformational changes of the enzyme.

2. Materials and methods

Malate synthase from baker's yeast was purified according to a new procedure [10]. A concentrated enzyme solution (c_{st}=15–20 mg/ml) was stored at 0–2°C in 5 mM Tris–HCl buffer, pH 8.1, containing 10 mM $MgCl_2$, 1 mM MgK_2EDTA and 0.2 mM dithiothreitol.

The enzymic assay makes use of the direct measurement of the rate of cleavage of the thio-ester bond of acetyl-CoA at λ=232 nm (cf. [3,4]). Buffers used in the optical tests contained 27 mM pyrophosphate–HCl, pH 8.1 or 27 mM Tris–HCl, pH 8.1. Tests were performed at 25°C or 4°C; they were started by addition of glyoxylate. Maximum specific activity of the enzyme in pyrophosphate buffer at 25°C (cf. [4]) was as high as 50–55 IU/mg. Enzyme concentration was determined spectrophotometrically using $A_{280nm}^{0.1\%,\,1cm}$=1.14.

Hydrodynamic and spectroscopic experiments were carried out at 4°C in the aforementioned 5 mM Tris–HCl storage buffer. To study the structural changes upon substrate binding, the following enzyme–ligand complexes were investigated: [enzyme·Mg^{2+}] [enzyme·Mg^{2+}·glyoxylate] [enzyme·Mg^{2+}·pyruvate] [enzyme·Mg^{2+}·acetyl-CoA] [enzyme·Mg^{2+}·acetyl-CoA·pyruvate] (cf. [9]).

Enzymic tests in the range of high dilution require the elimination of a number of artefacts (cf. [11]). The qualitative interpretation of the results obtained from hydrodynamic and spectroscopic measurements at low enzyme concentration may be used as unequivocal proof for dissociation or association. It is difficult however, to obtain quantitative results concerning the exact size or size distribution of the products formed at these low concentrations.

The study of small conformational changes upon ligand binding has to consider a number of sources

of error (cf. [12]). This holds especially for the determination of the small differences in the sedimentation coefficient. The influence of excess substrate (necessary for > 90% binding) on the sedimentation coefficient has to be taken into account. This was accomplished by performing runs at different concentrations of the substrates (corresponding to 90–99% binding).

Optical tests were measured in a Zeiss DMR 10 spectrophotometer. Sedimentation experiments were performed in a Beckman Model E analytical ultracentrifuge equipped with a high sensitivity photoelectric scanning system and a 10 inch recorder. Fluorescence analysis made use of a Hitachi Perkin-Elmer MPF-2A spectrophotometer. Circular dichroism spectra were recorded using a Roussel-Jouan Dichrographe II.

Data of independent measurements were collected and averaged. Experiments on samples from different preparations yielded identical results.

3. Results and discussion

3.1. *Concentration-dependent changes*

Enzymic tests were performed using different concentrations of the enzyme. The enzyme solution, stored at concentration c_{st}, was diluted to $15 \geqslant c_0 \geqslant 2.5 \cdot 10^{-4}$ mg/ml in Tris–HCl storage buffer at 0–2°C by careful weighing. Enzymic tests were carried out immediately after dilution. In general 10 μl (at the highest concentrations 1 μl) of the diluted enzyme solution of concentration c_0 were used in the test (final volume 1.5 ml). The concentration of the enzyme in the test therefore amounts to $1 \cdot 10^{-2} \geqslant c_{test} \geqslant 1.7 \cdot 10^{-6}$ mg/ml. Substrate and Mg^{2+} concentrations were kept constant; as taken from their binding constants [3,5], saturation of the enzyme with the substrates and Mg^{2+} does not change significantly in the concentration range under investigation.

In pyrophosphate buffer at 25°C (= standard condition for the optical test, cf. [4]) the specific activity decreased at a concentration $c_0 < 0.5$ mg/ml to reach a constant value of about 70% of maximum specific activity at lowest concentrations (fig. 1). Comparable deactivation profiles were obtained in pyrophosphate as well as in Tris buffer, both at 25°C and 4°C.

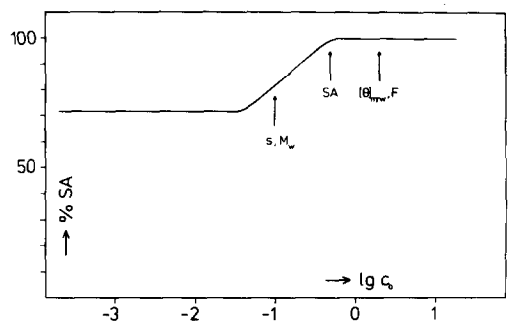

Fig. 1. Concentration-dependent changes of malate synthase. Specific activity SA (in %) decreased as a function of enzyme concentration c_0 (in mg/ml) brought into the optical test. The full line represents the results of the test performed in pyrophosphate buffer at 25°C. Spectroscopic and hydrodynamic investigations were carried out in Tris–HCl storage buffer at 4°C. Sedimentation velocity and high-speed sedimentation equilibrium ultracentrifugation as well as CD- and fluorescence spectroscopy show concentration-dependent changes of s and M_w or of $[\theta]_{mrw}$ and F respectively. The upper limit of the concentration c where the various parameters start changing are marked by arrows in the above-mentioned c_0-scale of the enzymic test.

For $c_{st} > 10$ mg/ml the deactivation profiles turned out to be essentially independent of the storage time ($t_{st} \approx 7$ days up to 3 months). Storage at c_{st} or $c_0 \leqslant 0.5$ mg/ml led to further deactivation at low c_0 with increasing storage time.

In order to correlate catalytic function and dissociation behaviour (cf. [13,14]) of the enzyme, ultracentrifugal analyses and spectroscopic experiments were performed under varying enzyme concentration c. Sedimentation velocity and high-speed sedimentation equilibria (cf. [15]) show concentration-dependent decreases of the sedimentation coefficient $s^{\circ}_{20,w}$ and the weight-average molecular weight M_w at $c < 0.1$ mg/ml. On the other hand the mean residue ellipticity in the far ultraviolet region, represented, e.g., by $[\theta]_{mrw,220nm}$ and the relative fluorescence intensity F of the enzyme at λ_{max} show significant changes at $c < 2$ mg/ml. All the (reduced) parameters decrease below a limiting value of enzyme concentration, obviously generated by partial dissociation. The result suggests the different experimental techniques to reflect changes in quaternary and/or tertiary structure of the enzyme with different sensitivity. The limiting concentration range where alterations of the native structure become detectable are indicated in fig. 1.

A striking feature of the given experimental results is the plateau value in the deactivation profile at low enzyme concentrations. It proves clearly that deactivation does not continue upon further dilution but leads to a definite final value where a rest-activity is maintained over several orders of magnitude of enzyme concentration. No clear-cut answer can be given so far concerning the quaternary structure and other properties of the enzyme in this range. It may equally represent active subunits or the enzyme after splitting of some fragments at low enzyme concentration. There is, however, clear evidence that the (partially) dissociated enzyme still shows catalytic activity. The existence of subunit activity has also been shown recently by reactivation studies with aldolase from rabbit muscle [16].

3.2. *Structural changes upon substrate binding*

Malate synthase has been investigated in the absence or presence of the substrates (acetyl-CoA, glyoxylate) or pyruvate as an substrate-analogue to glyoxylate. Since pyruvate is bound to the enzyme, but not converted to give a reaction product, investigation of the quaternary complex [enzyme·Mg^{2+}·acetyl-CoA·pyruvate] may be used as a tool to characterize the [enzyme·Mg^{2+}·substrate 1· substrate 2] complex.

Binding of the substrates or the analogue at concentrations c where dissociation does not take place, causes conformational changes of the enzyme. Ligand binding gives rise (a) to changes in the sedimentation coefficient $s^c_{20,w}$ (b) to shifts of the maximum of intrinsic protein fluorescence $\lambda_{em.,max}$ and (c) to changes of the molar ellipticity in the near ultraviolet region, represented, e.g., by decreases of $[\theta]_{295nm}$. The effects are summarized in table 1.

While glyoxylate and pyruvate induce comparable changes of all parameters under investigation, acetyl-CoA causes different overall effects. This is in accordance with the results of binding studies [5] which provided evidence for two independent binding

Table 1
Structural changes upon substrate binding, as measured by spectroscopic and hydronamic techniques

Method	CD-spectroscopy[a]	Fluorescence-spectroscopy[b]	Sedimentation analysis[c,d]
Substrate or analogue	$\Delta[\theta]_{295nm}$ (%)	$\Delta\lambda_{em.,max}$ (nm)	$\Delta s^2_{20,w}$ mg/ml (%)
Glyoxylate	−98.2 ± 5	−6.0 ± 0.5	−1.0 ± 0.3
Pyruvate	−70.6 ± 5	−6.5 ± 0.5	−0.9 ± 0.5
Acetyl-CoA	− 9.0 ± 5	+1.0 ± 0.5	+0.8 ± 0.2
Acetyl-CoA + pyruvate	−72.6 ± 5	−5.5 ± 0.5	+0.6 ± 0.4

[a] CD-spectra were recorded in tandem arrangement. The differences were derived from the smoothed spectra, e.g. {[enzyme·Mg^{2+}]/(buffer + glyoxylate)} minus {[enzyme·Mg^{2+}·glyoxylate]/buffer}. Molar ellipticity was calculated using M = 186 000 [9]

[b] Fluorescence emission spectra were recorded using $\lambda_{exc.}$ = 285 nm. The spectra show large shifts of the emission maxima after glyoxylate or pyruvate binding; unlike the sedimentation analyses, excess of substrates does not influence significantly λ_{max}

[c] To minimize systematic errors (e.g., due to temperature or rotor speed etc.) substrate-free enzyme solutions were used as a reference in each sedimentation run

[d] The s-values are influenced not only by conformational changes of the enzyme but also by the increase of particle weight and changes in preferential solvation caused by bound ligands. The above given Δs-values, therefore, represent the experimentally found overall effects at a given ligand concentration (8.9 mM glyoxylate, 8.9 mM pyruvate, 1.1 mM acetyl-CoA. Further details concerning this problem will be dealt with in a subsequent paper.

Tris–HCl storage buffer, 4°C. Effects induced by glyoxylate are close to those of the substrate analogue pyruvate, but different from those of acetyl-CoA. The absolute values for the substrate-free enzyme were found to be: $[\theta]_{295nm}$=8.4.10⁴ deg.cm².dmol⁻¹; $\lambda_{em.,max}$=331 nm; $s^2_{20,w}$ mg/ml= 8.32 S.

sites for the two substrates, acetyl-CoA and glyoxylate. On the other hand, the similar conformational effects, observed for glyoxylate or pyruvate, prove pyruvate to represent a good substrate analogue concerning the structural effects upon binding.

The fine-structure of the CD-difference-spectrum in the near ultraviolet region suggests that all three of the aromatic chromophores (phe, tyr, trp) are influenced by the formation of the quaternary complex (cf. [6]). Comparison of the trp-specific changes of $[\theta]$ at 295 nm shows that this chromophore is not influenced significantly by the binding of acetyl-CoA (in the absence of pyruvate). On the other hand, binding of glyoxylate or its analogue pyruvate causes a change of the ellipticity at this wavelength. The findings concerning trp-involvement are corroborated by the position of the fluorescence emission maximum which remains nearly unchanged upon binding of acetyl-CoA while glyoxylate or pyruvate binding induces strong blue shifts.

Small-angle X-ray scattering [9] and sedimentation analysis of malate synthase allow to detect changes of the overall structure of the enzyme which are reflected by changes in the values for the radius of gyration and the sedimentation coefficient of the order of about 1% after substrate binding. CD- and fluorescence-spectroscopy indicate larger alterations. However, they essentially reflect changes in the neighbourhood of intrinsic chromophores or fluorophores and do not give any information regarding changes in the overall structure of the enzyme molecule.

The observed changes in the gross structure of malate synthase upon substrate binding, at pH 8.1 and 4°C, are less pronounced as, e.g., in the case of glyceraldehyde-3-phosphate dehydrogenase where the cooperative binding of NAD^+, at pH 8.5 and 40°C, was shown to be accompanied by a 4% increase in the sedimentation coefficient [17] and a 5.4% volume contraction [18]. Compared with these large effects, malate synthase turns out to show only minor changes regarding its hydrodynamic and particle volumes, as indicated by sedimentation analysis and small-angle X-ray scattering [9].

On the other hand protein–ligand interactions of lactic dehydrogenase with NADH lead to changes of the sedimentation coefficient ($\Delta s \approx 3\%$) without detectable spectral effects as far as optical rotatory dispersion and circular dichroism are concerned [19]. Obviously the dynamic interplay of enzymes with their respective coenzymes or substrates is highly specific and varies considerably for a given enzyme and different ligands.

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