SMALL-ANGLE X-RAY STUDIES ON MALATE SYNTHASE FROM BAKER'S YEAST

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SUMMARY. Malate synthase was investigated in solution by the small-angle X-ray scattering technique. The substrate-free enzyme was shown to have a molecular weight of 186000, a radius of gyration of 3.96 nm, a maximum particle diameter of 11.2 nm, a volume of 343 nm^3, a radius of gyration of the thickness of 1.04 nm, and an axial ratio of 1 : 0.33. The enzyme molecule undergoes small changes in overall structure upon binding substrates. Investigation of the enzyme under prolonged exposure to X-rays led to an aggregation of the enzyme and allowed statements concerning the way of aggregation and factors influencing aggregation.

INTRODUCTION

Malate synthase (EC 4.1.3.2), the second enzyme of the glyoxylate bypass (1), catalyzes the synthesis of malate from acetyl-CoA and glyoxylate (2) thereby depending on Mg^{2+} as a cofactor (3,4). Physico-chemical properties of the enzyme from baker's yeast have been subject of earlier investigations (3, 5-9).

The present paper reports small-angle X-ray scattering studies on the enzyme and on enzyme-substrate complexes in solution. The same conditions regarding buffer, temperature, and specific activity were applied as used for the recent ultracentrifugal and spectroscopic experiments (9).

MATERIALS AND METHODS

Materials. Malate synthase was prepared from baker's yeast according to a new procedure (10). Thereby an electrophoretically pure and stable enzyme with a specific activity as high as 50-55 IU/mg was obtained. Enzyme concentration was determined spectrophotometrically or via dry-weight estimation (10). The specific volume was measured by use of a digital density meter (11).

A 5 mM Tris/HCl buffer pH 8.1, containing 10 mM $MgCl_2$, 1 mM MgK_2EDTA, 0.2 or 2 mM dithiothreitol (DTT), was used for all experiments. Measurements were carried out in the absence and presence of the substrates (acetyl-CoA, glyoxylate) or the substrate analogue pyruvate. Thus the following enzyme-ligand complexes were formed: [enzyme·Mg^{2+}], [enzyme·Mg^{2+}·glyoxylate], [enzyme·Mg^{2+}·pyruvate], [enzyme·Mg^{2+}·acetyl-CoA], [enzyme·Mg^{2+}·acetyl-CoA·pyruvate]. The degree of saturation of the enzyme with the various substrates or the analogue was 90-99 % in all experiments. No measurements have been performed in the absence of Mg^{2+}. Since Mg^{2+} a-

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lone does not change the structure of the enzyme, as follows from previous CD-measurements (6), the structure of the [enzyme·Mg^{2+}] complex may be regarded as representative for the native state of the enzyme.

Small-Angle X-Ray Scattering. The scattering experiments were performed using techniques, theory, and evaluation procedures described elsewhere (12-19). All samples were investigated at a temperature of 4°C. The concentration of the solutions varied from 2.5 to 60 mg protein per ml. Final data were collected and combined from measurements with samples from five different preparations.

The investigation of structural changes (cf. 20) requires that small changes of molecular parameters may be recognized with sufficient accuracy. Due to systematic errors in the course of measurement and evaluation, the absolute accuracy of the data might be much lower than the precision of their reproducibility. However, as far as only systematic apparative and numerical errors are involved, these are likely to be of the same magnitude and direction as long as all samples were investigated under identical or at least very similar conditions. This holds true e.g. for comparison of the unliganded and liganded enzyme or of differently liganded enzymes. Therefore, if we restrict ourselves to the evaluation of differences, these errors will probably cancel out and do not influence the reproducibility of results and statements concerning structural changes.

However, one has to make sure, that other systematic errors, which are neither apparative nor numerical, do not disturb the effects under investigation. Unfortunately this condition was not fulfilled a priori with malate synthase. The enzyme showed low stability against X-rays. Irradiation of the samples for several hours resulted in a continuous increase of the intensities in the innermost portion of the scattering curve. As will be shown below, this increase was caused by an X-ray induced aggregation of enzyme particles. Especially the scattering curves of the substrate-free enzyme were affected by aggregation phenomena to a large extent, whilst in the presence of substrates the systematic increase of intensity was less pronounced. To overcome this problem, the innermost portion of the scattering curve was measured repeatedly by subsequent short runs at low counting rates. The data from each run were evaluated separately. They were carefully checked for the absence of the above mentioned increase of intensities at the smallest angles. Only obviously unobjectionable data were used for the further evaluation, i.e. data were combined and averaged, extrapolated to zero angle and zero concentration and corrected for collimation effects. Due to these procedures we can be sure that our data for the enzyme in the absence or presence of substrates are not affected by the presence of high-molecular aggregates.

RESULTS

The Substrate-free Enzyme. From the scattering curve of the substrate-free enzyme (Fig.1) several molecular parameters were derived which are summarized in Table 1. The spherically averaged distance distribution, obtained by Fourier transformation of the entire scattering curve, yielded a maximum particle diameter D of 11.2 $\pm$ 0.6 nm. The radius of gyration R, determined both from the slope of the scattering curve at the smallest angles and from the distance distribution function, was found to be 3.96 $\pm$ 0.02 nm. Evaluation of the absolute value of the intensity scattered at zero angle yielded a molecular weight M of 186000 $\pm$ 3000; this result is based on an apparent isopotential specific volume of $\Phi' = 0.738\ cm^3/g$ obtained by precise density measurements. From the ratio of

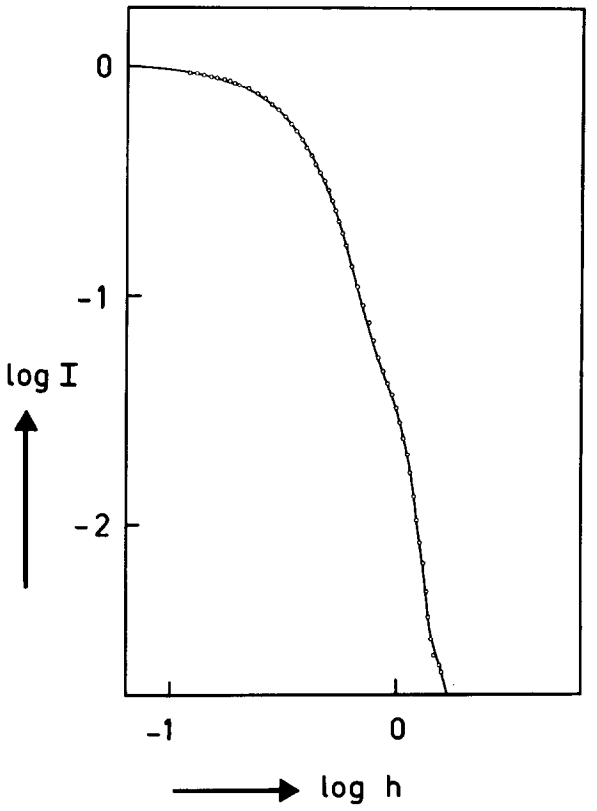

Fig.1: Scattering curve of malate synthase in the absence of substrates (o) and comparison with the theoretical curve for a circular cylinder of dimensions r = 5.4 nm and H = 3.6 nm (where $h = 4\pi \sin \theta/\lambda$, θ is half the scattering angle, and $\lambda = 0.154$ nm).

Table 1: Molecular parameters of the enzyme and of enzyme-substrate complexes

substrate or analogue	R (nm)	M	D (nm)	V (nm^3)	R_t (nm)
none	3.96	186000	11.2	343	1.04
glyoxylate	3.91		10.6	343	1.08
pyruvate	3.91		10.7	339	1.10
acetyl-CoA	3.93	188000	10.9		1.12
acetyl-CoA + pyruvate	3.91		10.9	346	1.05

the intensity scattered at zero angle to the value of Porod's invariant the particle volume V of 343 ± 5 nm^3 was obtained. This value is larger than the "dry volume", calculated from M and ϕ', by a factor of 1.53. This fact can be interpreted by the assumption of a solvation of 0.39 g water per g protein. The comparison of the experimental scattering curve with theoretical curves for various homogeneous geometrical bodies gave a very good fit for oblate circular cylinders. This is in best agreement with the finding of a thickness factor corresponding to a radius of gyration of the thickness R_t of 1.04 ± 0.06 nm. On the basis of the above data, a homogeneous circular cylinder of radius r = 5.4 nm and height H = 3.6 nm can be regarded as being equivalent in scattering to the substrate-free native enzyme (cf. Fig.1). Thus with an axial ratio of about 1 : 0.33 malate synthase obviously has a rather anisometric shape.

Enzyme-Substrate Complexes. The scattering curves for the various enzyme-substrate complexes were found to be very similar to the scattering curve for the enzyme in the absence of substrates. The results obtained therefrom are summarized in Table 1. The radii of gyration as well as the maximum particle diameters of these complexes are all slightly smaller than the values of the apoenzyme. The radii of gyration of the thickness, on the other hand, turned out to be slightly enlarged upon substrate binding. The volumes of the enzyme-substrate complexes were found to be essentially identical to the value for the apoenzyme within the limits of error. The experimentally found increase of the molecular weight upon binding acetyl-CoA is consistent with the actual increase of mass caused by this ligand.

X-Ray Induced Aggregation. As a consequence of the described changes in the scattering curves at small angles upon irradiation of the enzyme for several hours, it was found that both the radius of gyration and the molecular weight increase as a function of time. This clearly reflects the formation of high-molecular aggregates. In order to elucidate the way of aggregation and to show how the aggregation rate may be influenced, we set up a series of specially designed experiments. For this purpose we irradiated the samples for times essentially longer than in usual small-angle experiments.

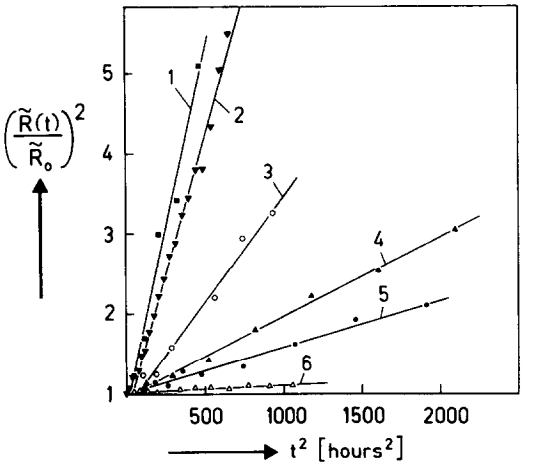

Fig.2: Plot showing the increase of the slit-smeared radius of gyration $\tilde{R}$ of substrate-free malate synthase with the time of irradiation t, in dependence on the integral primary intensity P_o (in arbitrary units; 1: 3.73, 2: 3.54, 3: 4.05, 4: 1.82, 5 and 6: 1.0) and the concentration of DTT (3 and 6: 2 mM, otherwise 0.2 mM).

An analysis of the scattering curves, taken at subsequent stages of aggregation, yielded retention of the thickness factor typical of disk-like particles. The radius of gyration of the thickness does not increase with proceeding aggregation. Moreover, so-called cross-section factors typical of rod-like particles could be observed after some time. From the distance distribution function the maximum particle diameter of the aggregates could be derived. It increases stepwise by amounts corresponding to about the diameter of the native enzyme particle.

The rate of aggregation was found to be a function of the intensity of incident radiation. Interrupts of irradiation are responded by interrupts of aggregation. A plot of the square of the slit-smeared radius of gyration $\tilde{R}$ versus the square of the irradiation time t proved to be a simple but useful tool for comparing different samples (Fig.2). The data obviously may be approximated by straight lines; their slopes representing a measure of the aggregation rate seem to depend exponentially on the intensity of incident radiation. In the presence of the substrates or the analogue or after adding excess DTT the rate of aggregation turned out to be considerably reduced.

DISCUSSION

X-ray investigations of enzymes in solution obviously may offer some difficulties concerning stability against X-rays. Malate synthase turned out to be very sensitive to X-irradiation. This problem was successfully overcome by some sophisticated precautions in the measurements as well as evaluation procedures. Therefore, the small differences in the molecular parameters found for the substrate-free enzyme and the various complexes between the enzyme and the substrates or the analogue may be interpreted as to reflect slight structural changes of the enzyme particle itself and not aggregation effects.

The apoenzyme was described to be a relatively anisometric particle as compared with other enzymes. This fact was established by comparison of the experimental scattering curve with theoretical ones as well as by the finding of a thickness factor and the maximum particle diameter. The binding of the substrates or the analogue to the apoenzyme obviously leads to structural changes accompanying the binding process. These changes were reflected by a decrease of the radius of gyration and the maximum particle diameter and a slight increase of the radius of gyration of the thickness. Similarly small changes of the overall structure were obtained by the sedimentation analysis (9). The decrease of the radius of gyration is also in analogy to the findings with glyceraldehyde-3-phosphate dehydrogenase (20) or pyruvate kinase (21) upon ligand binding.

A detailed investigation of the scattering behaviour of malate synthase under conditions strongly favouring aggregation allowed the establishment of a tentative model of this process. The retention of the original thickness factor and the finding of one, and later on of two cross-section factors, implies the first step of the aggregation to be the formation of a linear row of side by side associated enzyme particles, a process which is followed by a two-dimensional aggregation. The aggregation in the third dimension obviously does not occur during the time covered by our experiment.

The substrates and the analogue as well as DTT induced a stabilization of the en-

zyme against X-ray damages and subsequent aggregation phenomena. This may be explained by the action of these substances as scavengers of radicals formed by radiolysis or by protection of specific groups on the enzyme surface by these substances or indirectly by ligand-induced structural changes.

The application of the small-angle technique may be regarded as a promising contribution to the field of radiation biology. This study opens a new approach to the problem of irradiation-induced damages of enzymes by simultaneously using X-rays both for the production of damaged particles as well as their structural investigation. Small-angle X-ray scattering turned out to be a powerful tool for studying size, shape and structure of particles formed after irradiation as well as for investigating the kinetics of the aggregation process.

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