Dissociation of Catalase

A CORRELATION BETWEEN CHANGES IN SEDIMENTATION AND SPECTROSCOPIC PROPERTIES ACCOMPANYING DISSOCIATION OF BACTERIAL CATALASE IN ALKALINE SOLUTION

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1. At high concentrations, in 10mM-phosphate buffer, pH7.0, the sedimentation coefficient of bacterial catalase varies with concentration according to:

$$s_{20,w}^0 = s_{20,w} (1 - k_s c)$$

with $s_{20,w}^0 = 11.30S$ and $k_s = 6.29 \times 10^{-3} \text{ ml mg}^{-1}$. Sedimentation-equilibrium experiments yield a molecular weight of 240000. 2. Parallel studies of changes in sedimentation-velocity behaviour and in electronic spectra of bacterial catalase at $\text{pH} > 11$ were made. Dissociation is indicated by the appearance of a slow-moving (2.9S) component in sedimentation patterns and this is accompanied by marked changes in absorption spectrum in the Soret region. Values of $R = E_{406}/E_{355}$ show a theoretically predictable near-linear dependence on α , the degree of dissociation calculated from ultracentrifuge data. 3. The Soret absorption of bacterial catalase subunits is much lower than that of the native enzyme, and it is suggested that dissociation produces an environmental constraint on the prosthetic group that results in distortion of the porphyrin ring.

Recent kinetic work has suggested that the dissociation of catalase (hydrogen peroxide-hydrogen peroxide oxidoreductase, EC 1.11.1.6) is an important, and hitherto unrecognized, factor in the mechanism of catalase action at physiological pH, and that the meaningful assay of catalatic activity is a problem of considerable complexity (Jones & Suggett, 1968*a,b,c*; Brown, Jones & Suggett, 1969). The instability of ox liver catalase under more extreme conditions has been demonstrated in a number of studies (Sund, Weber & Mölbert, 1967; Samejima, 1959; Samejima & Yang, 1963). In most cases dissociation to M/4 subunits has been reported to occur, although it has been demonstrated that M/2 subunits are obtained by treatment with 8M-urea or 5M-guanidine hydrochloride in the absence of β -mercaptoethanol (aggregation of M/4 subunits via exposed thiol groups is presumed to have occurred in these cases). M/2 species have also been reported at low pH (Samejima & Yang, 1963) and after treatment with trypsin (Schütte, Steinbrecht & Winder, 1960). Important investigations by

Samejima and co-workers (Samejima & Yang, 1963; Samejima, McCabe & Yang, 1968) have shown that catalatically inactive ox liver subunits produced at high pH may be reconstituted to give an active native-like enzyme. Other workers have shown that catalatically inactive ox liver catalase subunits produced by alkaline treatment of the native enzyme have high peroxidatic activity (Anan, 1958; Inada, Kurozumi & Shibata, 1961; Caravaca & May, 1964; Caravaca, Dimond, Sommers & Wenk, 1967).

Catalase obtained from the bacterium *Micrococcus lysodeikticus* is a particularly valuable material for quantitative physicochemical studies, since the conditions of growth of the organism may be precisely controlled, the extraction procedure is straightforward and the preparations are highly reproducible, in terms of both purity and catalytic activity. The reported specific catalatic activity of bacterial catalase is considerably higher than that of any other known catalase (Herbert & Pinsent, 1948; Brill & Williams, 1961; Jones & Suggett, 1968*a*). Catalase from *M. lysodeikticus* was first crystallized by Herbert & Pinsent (1948), who measured the percentage of haematin present, by

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the pyridine haemochromogen method, and hence obtained a value of 232 000 for the molecular weight. This value (presumably) has been assumed in the reported molar extinction coefficients for bacterial catalase at 406 nm (Chance & Herbert, 1950; Maehly, 1961; Brill & Williams, 1961) and therefore these have been converted to give extinction coefficients expressed in terms of g of catalase/l. We have taken a value for E_{406} of $1.77 \text{ g}^{-1} \text{ cm}^2$ and have used this to estimate the catalase concentrations in our experiments. Cecil & Ogston (1948) made a brief sedimentation-velocity study using material supplied by D. Herbert & J. Pinsent and reported a value of 11.0S for 'S₂₀(corr.)'.

The present paper reports studies of the sedimentation characteristics of bacterial catalase under conditions (high concentration, neutral pH) where we believe (Jones & Suggett, 1968a) that catalase exists almost entirely in the native catalytically active form. These measurements formed an essential preliminary to the study of catalase dissociation, which was the main objective of our work. The second part of the paper reports studies of the dissociation of bacterial catalase in alkaline solution, and the results of sedimentation-velocity measurements are compared with observations of changes in the electronic spectrum of the solutions under the same conditions. These experiments offer the possibility of making some suggestions about structural changes in the region of the ferrihaem centres that accompany dissociation, and also afford a method of estimating the importance of dissociation under conditions where direct ultracentrifugal studies are impractical. The measurements also provide a comparison between the stabilities of ox liver catalase and bacterial catalase.

MATERIALS AND METHODS

Bacterial catalase was obtained from *M. lysodeikticus* (American Culture Collection no. 4698) and purified by a single $(\text{NH}_4)_2\text{SO}_4$ fractionation [fraction precipitated at between 40% and 60% saturation with $(\text{NH}_4)_2\text{SO}_4$ was retained] followed by ion-exchange chromatography on a Whatman DE-32 DEAE-cellulose column (with 50 mm-tris-HCl buffer, pH 7.5, in a 0–0.75 M-NaCl gradient), and finally by gel-filtration chromatography on Sephadex G-150 (10 mm-KH₂PO₄–Na₂HPO₄ buffer containing 0.1 M-NaCl). The purity measure (E_{406}/E_{280}) was 0.93. The specification of other materials used and the preparation of solutions have been described in an earlier paper (Jones & Suggett, 1968a). All experiments were carried out at 20°C.

Spectrophotometric measurements were made with an Optica CF 4 double-beam spectrophotometer; a Beckman model E analytical ultracentrifuge was used for sedimentation studies. Sedimentation-equilibrium experiments were run at speeds calculated from the equations of Van Holde & Baldwin (1958) for a 3:1 concentration

ratio between cell bottom and meniscus. Sedimentation velocities were measured at 59 780 or 50 740 rev./min by using schlieren optics, and sedimentation coefficients were calculated from the rate of movement of the point of maximum refractive-index gradient. Filled-Epon double-sector cells were used for measurements at high pH and run at 42 040 rev./min. In these experiments relative amounts of native and subunit catalase were estimated by measuring the areas under the schlieren peaks at a point where the subunit (slow) peak was clear of the meniscus. Concentrations were corrected for radial dilution where appropriate. In preliminary experiments we noted that the rate of formation of catalase subunits was markedly dependent on total catalase concentration and on pH. It is therefore difficult to estimate precisely the significance of the Johnston–Ogston effect in this case, but it is unlikely that the qualitative interpretation of the results is affected, and, indeed, for a globular protein like catalase the quantitative effect is probably small.

RESULTS AND DISCUSSION

Native bacterial catalase. Sedimentation-equilibrium studies were made by two methods to obtain a value for the molecular weight of bacterial catalase. Catalase at eight different concentrations was examined in two separate runs by using a short-column multicell technique, described by Yphantis (1960). From these experiments a molecular weight of $2.5 (\pm 0.1) \times 10^5$ was obtained, assuming a partial specific volume for bacterial catalase of 0.73 ml g^{-1} . Experiments with longer columns were also carried out with the Van Holde & Baldwin (1958) Method II. Three separate catalase solutions were employed with a column length between 3.0 and 4.0 mm. These studies yielded a molecular weight of $2.3 (\pm 0.1) \times 10^5$, again assuming a partial specific volume of 0.73 ml g^{-1} . Although the precision of these experiments could probably be improved, for example by the use of interference optics, for most purposes a value of 2.4×10^5 is an acceptable molecular weight for bacterial catalase. This value is in good agreement with results reported from studies of ox liver catalase (Sumner & Gralen, 1938; Samejima, 1959; Samejima & Yang, 1963; Sund *et al.* 1967) which have yielded molecular weight values in the range 2.4×10^5 – 2.5×10^5 .

Sund *et al.* (1967) have studied the concentration-dependence of the sedimentation coefficient of ox liver catalase and shown that this may be expressed by an equation of the form:

$$s_{20,w}^0 = s_{20,w}(1 - k_s c) \quad (1)$$

with $s_{20,w}^0 = 11.29\text{S}$, $k_s = 6.2 \times 10^{-3} \text{ ml mg}^{-1}$ and c is the catalase concentration in mg ml^{-1} . In Fig. 1 our results for bacterial catalase are compared with the concentration-dependence predicted by eqn. (1). In all experiments a single symmetrical peak was obtained. The results are well expressed by eqn. (1)

Fig. 1. Dependence on catalase concentration of the sedimentation coefficient of catalase in 10 mM-phosphate buffer, pH 7.0, containing 0.1 M-NaCl, at 20°C.

with the parameters $s_{20,w}^0 = 11.30\text{S}$ and $k_s = 6.29 \times 10^{-3}\text{mlmg}^{-1}$. Thus the ultracentrifugal characteristics of bacterial catalase from *M. lysodeikticus* are very similar indeed to those of ox liver catalase.

Dissociation of bacterial catalase in alkaline solution. At pH 12 bacterial catalase at a concentration of about 5mgml^{-1} ($\sim 20\mu\text{M}$) dissociates at a measurable rate. Two species were observed in sedimentation-velocity experiments, a fast component ($s_{20,w}$ 11.0S), which may be assigned as native catalase, and a slow component with $s_{20,w}$ 2.9S. The sedimentation coefficient of the slow component decreases further as dissociation approaches completion. This effect is most readily seen at the higher pH values; for example a $20\mu\text{M}$ -catalase solution after running for 24 h at pH 13 was found to contain 98% slow component with $s_{20,w}$ 2.0S. This decrease in sedimentation coefficient continued after the complete disappearance of the native form; in the above experiment a single peak with $s_{20,w}$ 1.2S was observed after 5 days. From these results it is clear that bacterial catalase is much more stable towards alkali than is ox liver catalase. Samejima (1959) and Samejima *et al.* (1968) have reported that the latter material is completely and rapidly ($<2\text{min}$) dissociated at pH 12, whereas our results indicate that a $20\mu\text{M}$ -bacterial catalase solution is only fully dissociated after 30 h at pH 13. Samejima *et al.* (1968) consider that the 2.85S subunit of ox liver catalase is a monohaem species. In our experiments the visible-light absorption increased at the 2.9S peak, in the direction of sedimentation, implying that the haem unit is retained in the subunits.

In order to make a comparison of changes in sedimentation properties and absorption spectra of alkaline catalase solutions, parallel ultracentrifuge and spectrophotometric experiments were per-

Fig. 2. Changes in the absorption spectrum of $1\mu\text{M}$ -catalase at pH 12.8 during a period of 20 h. Traces A-F correspond to degrees of dissociation (α) of 0.06, 0.21, 0.39, 0.62, 0.75 and 0.86 respectively.

formed. Experimental conditions were chosen such that the relative proportions of native and subunit species did not change significantly during an ultracentrifuge run.

The u.v.-visible spectra of alkaline bacterial catalase solutions show marked changes as dissociation proceeds (similar changes occur in acid and guanidine hydrochloride solutions). The most striking changes occur in the wavelength range 340–420 nm (Fig. 2), where the almost symmetrical Soret band of native catalase at 406 nm is gradually replaced by two overlapping bands with apparent maxima at about 355 and 392 nm. There is a clear isosbestic point at 369 nm. The appearance of subunits is accompanied by a large decrease in extinction coefficient at 406 nm and a small increase at 355 nm. For quantitative comparison the change in shape of the Soret band may be conveniently expressed in terms of the ratio (R) of the extinctions at 406 and 355 nm. This was considered more satisfactory than a single wavelength measurement because of the possibility that small changes in the total catalase concentration might occur in experiments of long duration.

Fig. 3. Variation of R with pH for $1 \mu\text{M}$ -catalase. $\circ$, Measurements made after 1 h; $\square$, measurements made after 20 h; $\triangle$, measurements extrapolated.

Fig. 4. Variation of R with α . The dotted line indicates a theoretical prediction based on eqn. (4).

Fig. 3 shows the variation of R with pH for $1 \mu\text{M}$ -catalase solutions at 1 h and 20 h after initiation of dissociation by changing the pH of the solution from that of the stock solution (10 mM-phosphate buffer, pH 7). Values of R obtained by extrapolation to zero time are also shown in Fig. 3. A noteworthy feature is the small increase in R that occurs in the pH range 7–11. Jones & Suggett (1968b) reported a similar small increase in catalase activity in this range, followed by a sharp decrease at pH > 11. Although both effects are small they suggest the possibility that changes in the configuration of the active site of catalase are occurring in this region that enhance both the 'sharpness' of the Soret band and the catalytic activity.

In Fig. 4 the spectroscopic changes are compared with the corresponding changes in sedimentation characteristics by plotting R against the degree

of dissociation (α) of the enzyme (computed from the weight-fraction distribution of fast and slow components). The correlation is satisfactorily represented by a straight line (solid line in Fig. 4) of the form:

$$R = 2.72 - 1.97\alpha \quad (2)$$

The dotted line in Fig. 4 is a theoretical curve computed according to the following considerations. Since (a) catalase solutions over the whole dissociation range show a clear isosbestic point (Fig. 2) and (b) in experiments where the total catalase concentration was carefully maintained over a large range of α both E_{406} and E_{355} were linearly dependent on α , we infer that only two species effectively contribute to the total extinction of a dissociating catalase solution. This suggests that the dissociation process may be represented as:

where N represents the native enzyme and S the subunits. Expressing R in terms of the extinction coefficients of native and subunit forms at 355 and 406 nm yields:

$$R = \frac{(E'_S - E'_N)\alpha + E'_N}{(E_S - E_N)\alpha + E_N} \quad (4)$$

where E' and E refer to the extinction coefficients per haematin at 406 and 355 nm respectively, and the subscripts S and N refer to the subunit and native forms of catalase. A plot of R against α would, according to eqn. (4), have a slope of:

$$\frac{dR}{d\alpha} = \frac{E_N(E'_S - E'_N) - E'_N(E_S - E_N)}{[(E_S - E_N)\alpha + E_N]^2} \quad (5)$$

It is clear from Fig. 2 that $(E_S - E_N) < E_N$, so that a near-linear dependence of R on α is predicted. The dotted curve in Fig. 4 was computed according to eqn. (4) by using the experimentally determined values of the extinction coefficients.

The nature of the spectroscopic change that accompanies the alkaline dissociation of catalase is very similar to that which accompanies the dimerization of protein-free ferrihaems (Brown & Lantzke, 1969; Brown, Dean & Jones, 1970). In both cases the extinction decreases markedly in the region of the Soret band maximum and a new band emerges at higher energies. In the latter case the spectral changes have been associated with distortions of the plane of the porphyrin ring in the formation of an oxo-bridged ferrihaem dimer (Brown, Jones & Lantzke, 1969). With haem enzymes it is not unlikely that changes in protein conformation and dissociation could produce significant environmental constraints on the con-

formation of the prosthetic group. If a precise conformation is a requirement for maximal activity, then structural changes of this type would be reflected in activity changes. Detailed studies of the absorption spectra of haem-enzymes have mainly related to the low-energy side of the Soret band. It seems possible that measurements on both the high-energy and low-energy sides of the Soret band may provide a useful probe of structure-activity relationships in these systems.

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