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The Catalase–Hydrogen Peroxide System

ROLE OF SUB-UNITS IN THE THERMAL DEACTIVATION OF BACTERIAL CATALASE IN THE ABSENCE OF SUBSTRATE

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1. Kinetic studies of the thermal deactivation of bacterial catalase in the absence of substrate suggest that the reaction involves a protonation-induced reversible dissociation of catalase into catalatically inactive sub-units, followed by an irreversible transformation of the sub-units into deactivated products. It is possible that the sub-units are mono-haem species. The rate of deactivation decreases with increasing pressure in accordance with the predictions of the proposed model. 2. The results also imply that the addition of hydrogen peroxide substrate induces the re-formation of active catalase. Under appropriate conditions the activity of catalase is found to increase with time in a manner that is quantitatively consistent with the results of deactivation studies.

It has been known for many years that solutions of catalase are more stable the more concentrated the solution. In preparative work the recommended optimum storage conditions are at concentrations of about 10mg./ml. (about 40μM) in phosphate buffer, pH7, and 0°. Using these conditions we have found that the activity of our bacterial catalase preparations did not change significantly over several months.

The rapid decrease in catalase activity at much lower concentrations has been discussed in terms of surface effects (i.e. the adsorption of catalase at interfaces), but there are few experimental data in the literature. Bonnichsen, Chance & Theorell (1947) considered this problem briefly and mentioned work of Agner on the effect of increasing the container surface area by the addition of glass beads. In their own experiments, however, no effect of changing (solution volume)/(container surface area) ratio was found, though this involved a comparison of data from flow experiments in which a solution/air interface was absent. We have found that changing the nature of the container surface from Pyrex glass to polythene had no effect on the rate of deactivation. The case for deactivation occurring by interfacial adsorption of catalase cannot be regarded as established. The major objective of our present work was the kinetic study of catalase action over the widest possible range of conditions, and we considered that a quantitative knowledge of the rate of deactivation of catalase in solution was desirable.

The range of catalase concentration over which the deactivation process may be studied is, at present, rather limited. As Bonnichsen *et al.* (1947) have shown, if the catalase concentration is too low the activity assay procedure becomes inaccurate. If the catalase concentration is too high there are severe manipulative difficulties in carrying out an activity assay. We have therefore restricted our measurements to the range of conditions that Bonnichsen *et al.* (1947) have shown to yield precise activity assays, remembering that bacterial catalase has an activity about twice that of horse erythrocyte catalase.

Attempts to study the effect of changing pH on the thermal deactivation process may be complicated by specific effects of buffer components. In the following discussion we use the term 'stock catalase solution' to imply a solution with a catalase concentration in excess of 1μM. In the assay procedure of Bonnichsen *et al.* (1947), a pH7 (phosphate buffer) stock catalase solution is diluted to about 1mμM with similar buffer containing hydrogen peroxide. If the stock catalase is diluted with buffers of a different composition and pH, the 'zero-time' catalase activity is unaltered, but the activity decreases with time at different rates, owing to differences in the rate of inactivation in the presence of substrate under these conditions. Thus, with this procedure, the maximal activity is substantially independent of pH (except under extreme conditions). We have confirmed this result, both with buffered solutions (see below) and with substantially unbuffered systems (P. Jones & A. Suggett, unpublished work), and it is supported

by the lack of pH-dependence of the rate constants, both for the formation of Compound I and the reaction of Compound I with reductants (Chance, 1952).

If, on the other hand, stock catalase solutions are prepared in different buffer systems of various pH and allowed to stand for some time, dilution to about 1mμM followed by rapid activity assay yields steady 'zero-time' activities that differ from the values found in phosphate buffer at pH 7, and that depend markedly on the nature of the buffer system. Similar effects are observed when the activity is measured at much higher catalase concentrations by the quenched-flow technique. If a stock catalase solution is diluted with buffer of similar composition and then allowed to stand before its activity is measured, a continuous decrease in activity with time occurs. It is this irreversible process that we have termed the 'thermal deactivation' of catalase.

MATERIALS AND METHODS

Materials. Bacterial catalase was obtained from *Micrococcus lysodeikticus* (American Type Culture Collection no. 4698) and purified by repeated fractionations with $(NH_4)_2SO_4$ and acetone, and dialysis against 10mM-KH_2PO_4–Na_2HPO_4 buffer, pH 6·8. The purity measure ($RZ = E_{407}/E_{280}$) was 0·85 for the samples used in this work. In later work we found that a purification procedure using ion-exchange chromatography on a DEAE-cellulose column, followed by gel filtration, yielded samples with RZ 0·95, which gave identical kinetic results. Catalase concentration was determined spectrophotometrically at the Soret band maximum (407mμ); the extinction coefficient at this wavelength was taken (Herbert & Pinsent, 1948; Brill & Williams, 1961) as ϵ_{407} $4{\cdot}10 \times 10^5$ M^{-1}cm.$^{-1}$.

The H_2O_2 was obtained as an 85% (w/w) unstabilized aqueous solution from Laporte Chemicals Ltd., Luton, Beds. Water was triply distilled. All other materials were of A.R. grade.

Grade-A burettes were used for iodometric titrations. For permanganate titrations a Radiometer automatic titrator was used. Pipettes were selected for reproducibility and calibrated. Agla micrometer syringes and B–D Cornwall automatic syringes were calibrated before use. Time was measured with a Favag precision timing unit. pH measurements were made with a Pye Dynacap pH-meter. Reaction were carried out in an air thermostat at 21°, except where otherwise stated.

Catalase activity measurements. The method used was a modification of that described by Bonnichsen *et al.* (1947). The latter method involved taking three samples from the reaction mixture by pipette within 30sec. of initiating the reaction. This technique is susceptible to volumetric error when the solution is evolving oxygen. Our modified procedure was as follows. First, 50ml. of 10mM buffer solution and 5ml. of approx. 0·1N-H_2O_2 were pipetted into a 250ml. beaker, and 5ml. of this solution was transferred to a 50ml. conical flask containing 5ml. of 5N-H_2SO_4 and, after the addition of 3ml. of 1% KI solution and 1 drop of saturated ammonium molybdate solution, was titrated with 0·01N-$Na_2S_2O_3$ solution with a 10ml. micro-burette. A 0·02ml. sample of catalase stock solution (in 10mM-phosphate buffer, pH 7) was accurately measured on to a small watch glass with the micrometer syringe. While the H_2O_2–buffer mixture was being magnetically stirred the watch glass was carefully dropped into the solution and the timer started simultaneously. After a selected time-interval, 10ml. of 5N-H_2SO_4 was injected into the stirred solution with a Cornwall syringe fitted with a wide-bore needle, and the timer was stopped on the first addition of acid. Two 5ml. samples of the resulting solution were transferred to separate 50ml. conical flasks, and KI and ammonium molybdate were added as before. The flasks were sealed with Parafilm before titration. The precautions necessary in the iodometric estimation of H_2O_2 in the presence of haemin and haemoproteins have been described elsewhere (Brown & Jones, 1968). The experiment was repeated several times with various reaction times and the catalase activity (k_B) was determined from the results by the procedure of Bonnichsen *et al.* (1947). The results are shown in Table 1. The values compare well with the literature [Brill & Williams (1961) give a Kat.f. that corresponds to $k_B = 6{\cdot}4 \times 10^7$ M^{-1}sec.$^{-1}$]. The errors quoted are the maximum deviations from the means. k_B was found to be independent of pH in the range 5·3–9·5.

Table 1. *Dependence on* pH *of the activity of bacterial catalase as determined by a modification of the method of Bonnichsen* et al. (1947)

The temperature was 21° and the initial enzyme concentration ($[E_0]$) was 0·640mμM.

pH	$10^{-7}k_B$ (M^{-1}sec.$^{-1}$)	pH	$10^{-7}k_B$ (M^{-1}sec.$^{-1}$)
5·34	6·45±0·1	7·80	6·41±0·1
6·29	6·35±0·1	8·26	6·45±0·1
7·00	6·45±0·1	9·49	6·35±0·1

Kinetics of thermal deactivation. Stock catalase solutions were prepared in a variety of buffers at different pH values in the range 5·3–9·7 and kept overnight in the air thermostat at 21°. Deactivation was initiated by adding, with magnetic stirring, the appropriate volume of stock catalase solution to 50ml. of a 10mM buffer solution that had the same composition as the stock buffer. After a measured period of time the catalatic reaction was initiated by injecting 5ml. of about 0·1N-H_2O_2 into the mixture, the timer being started after 2·5ml. had been added. After a selected catalatic reaction time, the reaction was quenched and analysis carried out by the procedure described in the preceding section. We define k_t as the observed first-order rate constant for the catalatic reaction, measured after a deactivation time t, divided by the stoicheiometric catalase concentration; k_0 is the activity (as defined by Bonnichsen *et al.* 1947) of catalase that has been equilibrated as a stock solution with a particular buffer at fixed pH and is obtained by extrapolation of the values of k_t to the instant at which deactivation was initiated.

RESULTS AND DISCUSSION

Fig. 1 shows that k_t initially varies linearly with time and at different rates depending on the

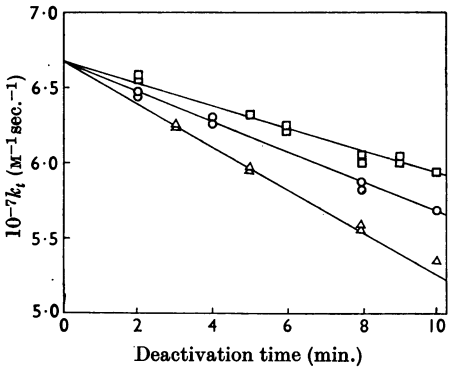

Fig. 1. Dependence of k_t on deactivation time in 10mM-phosphate buffer, pH 7·00, at 21°. □, $[E_0]$ 0·906mμM; ○, $[E_0]$ 0·604mμM; △, $[E_0]$ 0·3624mμM.

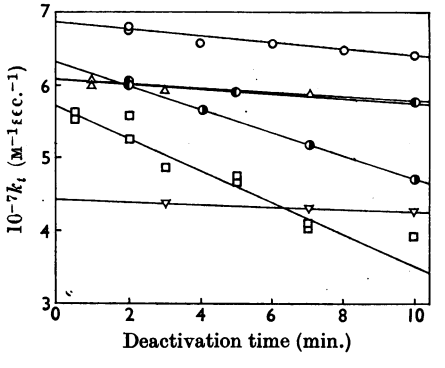

Fig. 2. Dependence of k_t on deactivation time at 21° with $[E_0]$ 0·604mμM. □, pH 5·34 (citric acid–Na_2HPO_4); ◑, pH 6·16 (citric acid–Na_2HPO_4); ○, pH 8·26 (KH_2PO_4–Na_2HPO_4); ◐, pH 9·04 (Na_2CO_3–$NaHCO_3$); △, pH 9·56 (Na_2CO_3–$NaHCO_3$); ▽, pH 9·66 (borax–Na_2CO_3).

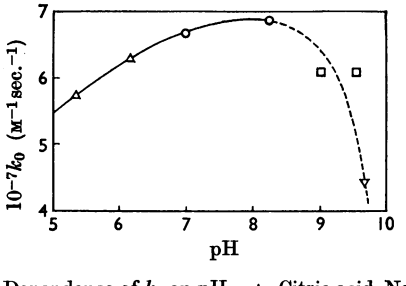

Fig. 3. Dependence of k_0 on pH. △, Citric acid–Na_2HPO_4 buffers; ○, KH_2PO_4–Na_2HPO_4 buffers; □, Na_2CO_3–$NaHCO_3$ buffers; ▽, borax–Na_2CO_3 buffer.

stoicheiometric catalase concentration. k_0 is independent of catalase concentration. Fig. 2 shows the results for different buffers at fixed catalase concentration. In Fig. 3 the values of k_0 are plotted against pH and show that there are

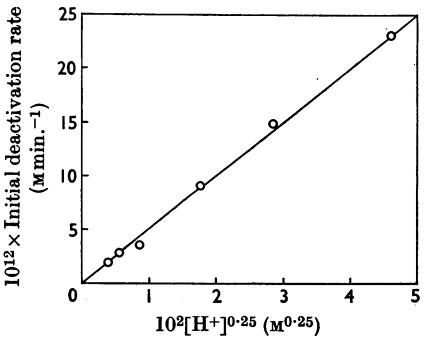

Fig. 4. Variation in initial deactivation rate with $[H^+]$.

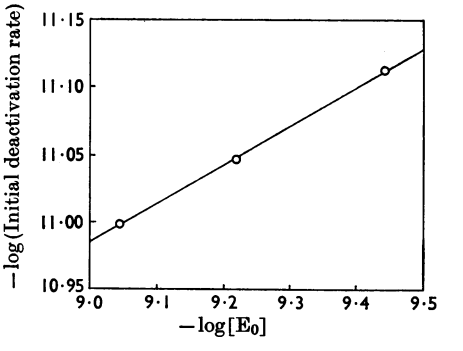

Fig. 5. Plot of log (initial deactivation rate) against log$[E_0]$.

significant specific buffer effects; it is noteworthy that k_0 in phosphate buffer at pH 8·26 is higher than k_B. The slopes of the lines in Figs. 1 and 2 represent the initial rates of deactivation under various conditions and we define the reaction rate in terms of the time derivative of the active catalase concentration by the expression:

$$d[E_a]/dt = ([E_0]/k_0) \cdot dk_t/dt \qquad (1)$$

where $[E_a]$ and $[E_0]$ are the active and stoicheiometric catalase concentrations respectively. Fig. 4 shows that $-d[E_a]/dt$ varies smoothly and monotonically with H^+ ion concentration at fixed catalase concentration (in contrast with the values of k_0) so that it appears probable that specific buffer effects are absent. Fig. 5 shows a logarithmic plot for the dependence on catalase concentration, over the rather small accessible range. Combining the two results, the following rate law satisfactorily describes the data:

$$-d[E_a]/dt = k[E_0]^{0.28}_d[H^+]^{0.25} \qquad (2)$$

Fractional reaction orders can arise in rate laws in a variety of ways. For this reaction we have the

additional kinetic information that catalase solutions are stable at high concentrations and this result, together with eqn. (2), strongly suggests that the thermal deactivation reaction involves a preliminary dissociation of the catalase molecule. Recalling that catalase is a tetranuclear ferric haemoprotein, we consider the following simple reaction scheme, which incorporates the idea of a reversible protonation-induced dissociation of catalase into four mono-haem sub-units. The sub-units are considered to have insignificant catalatic activity and can undergo an irreversible change that leads to an overall irreversible thermal deactivation of catalase. This model may be represented as:

$$(\text{Haem})_4 + \text{H}^+ \underset{}{\overset{K_D}{\rightleftharpoons}} 4\ (\text{Haem}) \qquad \text{(i)}$$

$$(\text{Haem}) \xrightarrow{k'} \text{Deactivated product} \qquad \text{(ii)}$$

where $(\text{Haem})_4$ represents catalase. Under conditions where the equilibrium concentration of sub-units is a small fraction of the total catalase concentration this model yields the rate law:

$$-\text{d}[\text{E}_a]/\text{d}t = k'K_D[\text{E}_0]^{0\cdot25}[\text{H}^+]^{0\cdot25} \qquad (3)$$

which agrees reasonably well with the experimental rate law (eqn. 2).

Even if the dissociation model is conceptually valid it would be unrealistic to suppose that, in the simple form given above, it would apply at all catalase concentrations. By analogy with the dissociation of haemoglobin, the occurrence of partially dissociated species would be expected under some conditions. Nevertheless, quantitative calculations based on the simple model provide an illustration of its implications and prove useful in further tests. From the data we calculate, by successive approximations, a value for K_D of about 10^{-22}M^2 at pH 7 and 21° and hence the fraction of catalase present as catalatically active tetramer at equilibrium at various stoicheiometric catalase concentrations: for stoicheiometric catalase concentrations of 10, 1, 0·1 and 0·01 mμM, the percentage of catalase present as tetramers is calculated as 98, 92, 56 and 1·5 respectively. Fig. 6 shows the variation with temperature of k_0 and $-\text{d}[\text{E}_a]/\text{d}t$, with a stoicheiometric catalase concentration of 0·6 mμM in 10 mM-phosphate buffer, pH 7. From the latter data we may estimate the stability of catalase solutions of different concentrations under normal storage conditions, by calculating a 'half deactivation time', $t_{\frac{1}{2}}$, based on the initial deactivation rate, obtained by using $K_D = 10^{-22}$M^2: for stoicheiometric catalase concentrations of 1, 10 and 40 μM (the last is equivalent to 10 mg./ml.), $t_{\frac{1}{2}}$ (0°) is estimated to be 8 months, 2 years and 6 years respectively. The results shown in Fig. 6

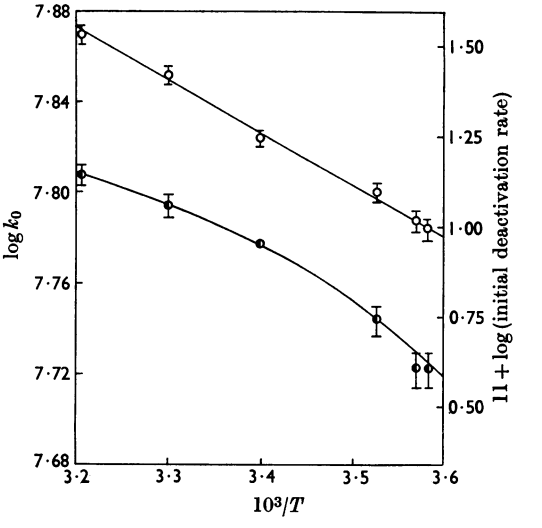

Fig. 6. Temperature dependencies of k_0 and $-\text{d}[\text{E}_a]/\text{d}t$; ○, $\log k_0$ against $1/T$; ◐, $\log$(initial $-\text{d}[\text{E}_a]/\text{d}t$) against $1/T$.

yield an overall activation energy of $1\cdot01 \pm 0\cdot06$ kcal.mole^{-1} for the catalatic reaction, in good agreement with the literature.

The formation of catalase sub-units has been demonstrated at very high pH (Samejima, 1959*a*,*b*) and after freeze-drying (Tanford & Lovrien, 1962). A liver catalase sub-unit of insignificant catalatic activity has been shown to possess powerful peroxidatic properties both *in vitro* (Caravaca & May, 1964) and *in vivo* (Caravaca, Dimond, Sommers & Wenk, 1967). Jones, Pain & Suggett (1968) have shown, by sedimentation-velocity studies in the ultracentrifuge, that bacterial catalase Compound II is a sub-unit species with a molecular weight of about 5×10^4. This result provided the first indication that sub-unit species might be involved in the mechanism of catalase action at physiological pH *in vitro*. From our present kinetic data it is clear that significant dissociation of catalase would not be expected under conditions where direct ultracentrifugal studies are possible. It was therefore necessary to consider alternative ways in which the dissociation model for the thermal deactivation of catalase might be tested.

Effect of pressure on thermal deactivation. Catalase is a globular protein and it is likely that dissociation into sub-units would lead to an increase in volume. The irreversible deactivation of the sub-unit would probably involve conformational changes in the polypeptide chains, leading to an increased volume in the transition state. Overall, the dissociation model would predict a substantial positive volume of activation for the thermal deactivation reaction,

Table 2. *Dependence on pressure of the rate of deactivation of bacterial catalase*

The initial enzyme concentration ([E_0]) was 0·604 mμM. Expt. I yields:

$$\frac{\text{Deactivation rate at 1 atm. at 21°}}{\text{Deactivation rate at 2550 atm. at 24°}} = 1{\cdot}6$$

Expt. II yields:

$$\frac{\text{Deactivation rate at 1 atm. at 23°}}{\text{Deactivation rate at 2550 atm. at 21°}} = 2{\cdot}7$$

Expt.	Time at > 1000 atm. (min.)	Time at < 1000 atm. + time at 1 atm.	Total time (min.)	Lab. temp.	$10^{-7}k_t$ (M^{-1} sec.$^{-1}$)
I	155	40	195	24°	3·05
	—	195	195	21	2·10
	—	40	40	21	4·60
II	175	30	205	21	3·61
	—	205	205	23	1·40
	—	30	30	21	4·90

which implies that the process may be retarded by the application of high pressure to the solution. Though precise models for the proposed deactivation at interfaces have not been formulated, it is unlikely that the adsorption of catalase as a monolayer would be accompanied by a significant increase in volume.

We have studied the effect of pressure on the thermal deactivation of catalase in the following manner. Stock catalase solution (in 10 mM-phosphate buffer, pH 7) was diluted to the required concentration with buffer of similar composition and placed in a polythene bag. Air was squeezed out of the bag, which was then tied tightly and placed in a 'bomb' that contained silicone oil as the pressurizing fluid. The pressure was increased as quickly as possible until a pressure of 2550 atm. was attained. The catalase solution was left under this pressure for about 3 hr. After release of the pressure, the polythene bag was removed, cleaned and dried, after which the catalase solution was extracted and its activity assayed as described above. A blank run at 1 atm. was carried out simultaneously, the same conditions (apart from pressure) being maintained as rigorously as possible. The results are shown in Table 2. The average rate of deactivation at 1 atm. is about twice that at 2550 atm. Since in the high-pressure experiments the large increase in pressure is likely to increase the temperature of the solution by about 10° initially, our result probably underestimates the pressure effect on the deactivation process. The results clearly indicate an appreciable positive volume of activation for the thermal deactivation process.

Effect of time on catalase activity. We have found the activity of bacterial catalase, under fixed conditions of buffer composition, pH and temperature, to be independent of catalase concentration between 0·1 and 10 mμM (for the higher concentrations a quenched-flow method was used; P. Jones & A. Suggett, unpublished work). From our estimated value of K_D, at equilibrium 44% of the catalase should be present as sub-units at 0·1 mμM whereas at 10 mμM only 2% of sub-units should be present. In the light of these results the dissociation model for thermal deactivation can only be maintained with the aid of the further assumption that the addition of hydrogen peroxide substrate brings about a re-formation of active tetrameric catalase from sub-units. This suggests that measurements of the change in the catalatic activity of dilute catalase solution with time, at short reaction times, might reveal an increase in activity with time, associated with the tetramer re-formation process.

This idea receives some support from results reported by George (1949) for the variation with time of the rate of evolution of oxygen (measured manometrically) in the catalatic reaction with a low concentration of ox liver catalase. At peroxide concentrations below 40 mM the rate was found to increase at first before gradually decreasing. At higher peroxide concentrations no initial rise was observed. George (1949) does not comment on these results and they are ignored in the equation proposed to describe the variation of the reaction rate with time. It seems possible that the low rates at short times were considered an artifact of the manometric technique.

We have carried out experiments using the titrimetric assay procedure. It is necessary to employ a suitably low catalase concentration and, since it is likely that the rate of the presumed re-formation reaction will increase with hydrogen peroxide concentration, a low substrate concentration consistent with the maintenance of analytical accuracy. Stock catalase (in 10 mM-phosphate buffer, pH 7) was diluted with similar buffer and allowed to stand for 30 sec. before initiation of the catalatic reaction. The initial hydrogen peroxide

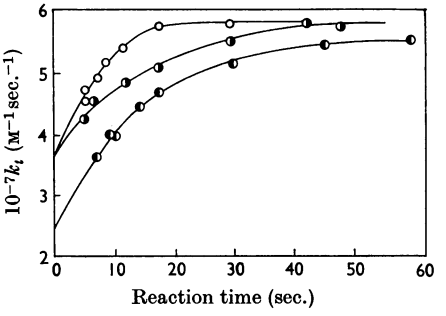

Fig. 7. Variation in catalase activity with reaction time in 10mM-phosphate buffer, pH 7·00, at 21°. ○, $[E_0]$ 0·18mμM, $[H_2O_2]$ 10mM; ◑, $[E_0]$ 0·18mμM, $[H_2O_2]$ 2mM; ◐, $[E_0]$ 0·09mμM, $[H_2O_2]$ 2mM.

concentration was between 2 and 10mM and it proved possible to use catalatic reaction times as short as 5sec. After quenching, the hydrogen peroxide was estimated by permanganate titration (2–10mN-potassium permanganate) in the automatic titrimeter. Values of the activity k_t were calculated for each reaction time. The results (Fig. 7) clearly show the predicted increases of activity with time. A quantitative test may be made by extrapolating the activities to zero reaction time; the intercept yields a value of the fraction of the stoicheiometric catalase concentration present as active tetramer and hence a value of K_D. These experiments yield $K_D = 4 \times 10^{-22}M^2$, in excellent agreement with the value obtained previously.

Conclusions. Though a contribution to the thermal deactivation of catalase by adsorption at interfaces cannot be ruled out, our results show that the major effects arise via a reversible dissociation of catalase into catalatically inactive sub-units. Dissociation is induced by protonation of catalase and the association–dissociation process appears to be a rather mobile equilibrium. Since catalase is a tetrameric ferric haemoprotein and our results suggest that catalase dissociates into four sub-units, it seems reasonable to suppose that the sub-units are mono-haem species, though this is not proved and is not required by the tests of the dissociation model that we have made.

Addition of substrate to a catalase solution that contains an appreciable proportion of sub-units produces two opposing effects: (i) re-formation of tetrameric catalase leading to increased catalatic activity; (ii) inhibition of catalase activity in the presence of substrate. If process (ii) proceeds appreciably before (i) is complete, maximal catalatic activity will not be exhibited. This suggests an explanation for the low activities typically obtained in assay procedures with very low catalase concentrations.

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