A Study by Rotatory Dispersion of the Denaturation of Catalase and Peroxidase

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The recent applications of rotatory dispersion techniques to the study of protein structure have for the most part utilized the effect of protein conformation upon dispersion curves that are associated with ultraviolet absorption bands (1-7). The hemoproteins are unique among biologically important proteins in that they also have chromophores in the visible range of the spectrum. It has been demonstrated, in the case of cytochrome c, that these chromophores can produce a Cotton effect in the visible region (8). It is shown in the present study that catalase and peroxidase also exhibit a Cotton effect, but only under conditions in which the proteins have ordered structures. Consequently, these dispersion curves have been used in a study of the denaturation of hemoproteins with acid and base, as well as in a study of the removal of heme from catalase and the recombination of heme with the resulting apoprotein.

EXPERIMENTAL PROCEDURE

Reagents and Materials—Beef liver catalase was obtained as a crystalline suspension in ammonium sulfate from the Sigma Chemical Company. The concentrated solutions required for rotatory dispersion measurements were prepared by the following procedure. To 10 ml of the catalase suspension in a dialysis bag were added 3 to 4 drops of 0.5% phosphate buffer, pH 7.5; the mixture was dialyzed against distilled water for 12 hours with frequent changes; dialysis was then continued against the phosphate buffer for 2 additional hours. The dialysate was centrifuged at 7000 r.p.m. for 15 minutes at 3°, and the white precipitate of denatured protein was removed. The green solution, pH 7.5, was refrigerated and used as soon as possible for the rotatory dispersion measurements and for the heme removal reactions. For the rotation measurements at pH 11.0, an aliquot of the pH 7.5 solution was cooled at 3° in an ice bath, and the pH was brought to 11.0 with cold sodium hydroxide solution. For the rotation measurements at pH 2.6, another aliquot was similarly treated with hydrochloric acid.

Horseradish peroxidase in powdered form from the Sigma Chemical Company was dissolved in distilled water, yielding a pH 7.2 solution, which was used directly for the rotatory dispersion measurements; the pH 11.0 solution was prepared by the addition of sodium hydroxide.

The activities of catalase and peroxidase were determined according to the procedure outlined by Chance and Macnab (9) and originally devised by Chance and Herbert for catalase (10, 11) and by Devlin for peroxidase (12).

The pH 7.5 buffer solution was prepared from Fisher reagent grade Na2HPO4·7H2O and KH2PO4. Crystalline heme was from the California Corporation for Biochemical Research. The dialysis tubing, from the Visking Corporation, was treated with hot saturated sodium bicarbonate solution before use.

Instrumentation—The rotatory dispersion curves were recorded by means of a Rudolph photoelectric spectropolarimeter, model 200 S/80Q/650. The solutions were placed in a 1-cm quartz cell, and rotations were determined at 25°-26°. (Apocatalase, however, was kept at 0° throughout the measurements by circulating water from a constant temperature bath through a jacket surrounding the polarimeter tube compartment.) The light source was a zirconium arc.

The measurements were not easy to carry out, since the amount of light transmitted through the solution in the region of the absorption band was small. In order to overcome this difficulty, the rotations were determined with a slit width of 1.5 mm. Consequently, each point in the dispersion curve must be considered an average value of the rotation over a wave length range at a given position in the spectrum. The observed rotations of the curves plotted in Figs. 1, 5, and 7 ranged from zero to a maximum of −0.15°. The reproducibility of a rotation at a given wavelength was not good; the values were generally accurate to ± 0.01°. Nevertheless, the shapes of the curves, i.e., the positions of peaks, troughs, and inflection points, were always reproducible. The rotatory dispersion curves plotted in the figures were obtained by averaging the rotation values at each wave length for several curves, generally five, obtained on different days with different solutions, in some cases at different concentrations. None of the experimental curves differs from the average curves in its shape, and since the shapes of the curves, rather than absolute values of rotation, are pertinent in this investigation, the data are considered adequate.

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1 We have been informed, in a recent communication from Mr. Peter Urnes, in the laboratory of Professor Paul Doty of Harvard University, of the discovery, with solutions of high optical density, of rotatory dispersion artifacts that are explained by the relatively high proportion of stray light of wave lengths other than the monochromator-set wave length reaching the analyzer. We are grateful to Mr. Urnes for sending us this information.
Spectra were obtained with a Cary model 14 spectrophotometer, and single wave length absorptions with a Beckman model DU spectrophotometer with 1-cm absorption cells. Concentrations of catalase were calculated by using a millimolar extinction coefficient of 10.8 at the absorption maximum of 620 m\(\mu\) (13). Peroxidase concentrations were similarly based upon an extinction coefficient of 12 at 640 m\(\mu\) (14, 15).

Sedimentation constants were determined with a Spinco model E analytical ultracentrifuge equipped with Epon centerpiece cells, thermistor temperature control, and phase plate optical system. The temperature was controlled between 1.5 and 2.5°. The solutions for these determinations contained from 0.2 to 0.6% catalase or peroxidase and were made up by dilution of the solutions used for the optical rotation experiments.

pH was measured with a Beckman model G pH meter.

Removal of Heme from Catalase—Since the preparation of pure globin from hemoglobin was reported by Rossi-Fanelli, Antonini, and Caputo (16), their method has been applied in the preparation of apocatalase.

One volume of cold 1 to 3% catalase solution was added dropwise with vigorous stirring at -20° to 20 to 30 volumes of acetone containing 5 ml of 2 N HCl at -20° for 10 to 20 minutes with occasional stirring; the almost colorless protein precipitate was collected by centrifuging for 15 minutes at 2000 r.p.m. at -20° in a Spinco model L preparative ultracentrifuge. The precipitate was dissolved in cold distilled water and dialyzed against distilled water to remove the acetone. The contents of the dialysis sack were centrifuged at 2000 r.p.m. for 15 minutes, and the solution was stored at 3° for use in the recombination experiments.

Theale (17) has recently reported the isolation of apocatalase by a procedure using methyl ethyl ketone, instead of acetone, in which ice bath temperatures could be employed. Theale's procedure was repeated, thus making available samples of apocatalase prepared by two different techniques.

Recombination of Apocatalase with Hematin—The following procedure was employed to study the stoichiometry of the reaction of apocatalase with hematin. A solution of hematin was prepared by dissolving a quantity of crystallo hemin to give a final concentration of 4.4 \(\times\) \(10^{-4}\) M in the smallest possible quantity of 0.1 N sodium hydroxide and by diluting with water. The resulting solution, whose pH was 9.0, was added to 4.4 \(\times\) \(10^{-4}\) M solutions of apocatalase2 prepared by each of the methods described above, and the reaction was followed by spectrophotometric titration at 405 m\(\mu\). A 1-ml sample of apocatalase was placed in the spectrophotometer cell, which was kept at 1° by allowing water from a 3° bath to flow through Thermosponors surrounding the cell compartment of the spectrophotometer. Each stepwise addition of hematin was made by placing 10 \(\mu\)g of the hematin solution on a stirring rod and mixing it rapidly with the apocatalase. The resulting solution was permitted to equilibrate for 5 minutes before an optical density reading was made; a control curve was obtained by repeating the above experiment with 1 ml of water replacing the apocatalase solution.

The spectra and rotatory dispersions of "recombined catalase" were determined on solutions containing 4 moles of hematin per mole of catalase.

RESULTS AND DISCUSSION

pH and Rotatory Dispersion of Catalase—The molecule of catalase (19) consists of four polypeptide chains, each of which presumably includes one heme. Tanford and Lovrien (20) have shown, by using ultracentrifugation and viscosity data, that the molecule is stable in the pH range between 3.5 and 10.5, but that it dissociates into quarters outside this range.

The sample of catalase used in the present study gave \(s_{20,\omega} = 11.2\) S and an activity of 2.0 \(\times\) 10\(^4\) liters per mole second, compared with \(s_{20,\omega} = 11.6\) S and an activity of 1.7 \(\times\) 10\(^4\) liters per mole second reported for intact catalase by Tanford and Lovrien. The rotatory dispersion curve of this preparation (Fig. 1A) exhibited a Cotton effect with an inflection at 610 m\(\mu\), thus approximating the position of the absorption peak at 620 m\(\mu\).

When titrated to pH 11, the catalase solution gave \(s_{20,\omega} = 3.6\) S, corresponding to Tanford's quarter molecule. This solution had no measurable activity at concentrations in the vicinity of 1%. At pH 2.8, \(s_{20,\omega} = 2.0\) S and the activity was 7.3 \(\times\) 10\(^5\) liters per mole second. The spectra of the catalase solutions at neutral, acid, and alkaline pH are compared in Fig. 2. It will be noted that they are very much alike, especially in their retention of the peak of 620 m\(\mu\). The similarity of the spectra at pH 7.5 and pH 11 is particularly striking.

In spite of this spectral similarity at different pH values, the rotatory dispersion curves are substantially different. At pH 11 the Cotton effect was completely eliminated (Fig. 1B),3 an approximate \(\lambda_c\) value could, in fact, be calculated by means of a Drude plot (1/[\(\alpha\)] versus \(\lambda^2\)) of the data (\(\lambda_c \approx 210\) m\(\mu\)).

It thus appears that the asymmetry associated with the 620

3 That the observed Cotton effects are not artifacts is again indicated, if not proved, by the fact that solutions with almost identical absorption spectra do not show this effect.

4 The points in a Yang-Doty plot (\(\lambda^2\) [\(\alpha\)] versus [\(\alpha\)] (2) are too close together and too far from the intercept to permit \(\lambda_c\) determination by this means.
Rotatory Dispersion of Catalase and Peroxidase

**Fig. 2.** Spectra of catalase (1.5% solutions). A, pH 7.5; B, pH 11.0; C, pH 3.0.

**Fig. 3.** Rotatory dispersion of catalase, pH 2.8, 1.5%.

**Fig. 4.** Possible orientation of hemes in catalase.

**Fig. 5.** Rotatory dispersion of peroxidase. A, pH 7.2, 2.5 to 3.5%; B, pH 11.0, 3.0%. Estimated reproducibility is indicated as in Fig. 1.

An alternate explanation (Hypothesis II) follows from the work of Blout and Stryer (21), who have demonstrated that a complex between protein or polypeptide in the helical conformation with a dye, such as acridine orange, exhibits a Cotton effect in the absorption band of the dye; on the other hand, no Cotton effect can be observed when the protein or polypeptide is in the random coil conformation. Apparently, combination with the helical polypeptide confers asymmetry upon the otherwise symmetrical dye molecule. It is possible that the heme in catalase can be an asymmetrical center for the same reason that the dye shows asymmetry in combination with polypeptides in the Blout-Stryer experiments.

**pH and Rotatory Dispersion of Peroxidase**—Experiments with peroxidase offer an opportunity to test these two hypotheses experimentally. Unlike catalase, peroxidase contains only one heme per molecule (22). It may be supposed, therefore, that changes in pH will not result in a cleavage of the peroxidase molecule. If the presence or absence of a Cotton effect can be attributed to unsymmetrical or symmetrical attachment of the protein moiety (Hypothesis I), then peroxidase should have similar rotatory dispersion at all pH values. Either both sides of the heme look alike, yielding “plain” dispersion, or they look different, producing a Cotton effect; pH should not change the picture in either case. It does not appear possible, at any rate, to convert an unsymmetrical structure into a symmetrical one, according to the scheme of Fig. 4.

If, on the other hand, the presence or absence of the Cotton effect is analogous to the Blout-Stryer observations, then pH changes, or any changes in conditions that would affect the conformation of the protein, should produce changes in rotatory dispersion similar to those observed for catalase.

Peroxidase was studied at pH 7.2 and pH 11.0. The anticipated lack of change in the molecular weight was borne out by a relatively slight change in sedimentation constant: $s_{w, 0} = 3.61$ S at pH 7.2 and 3.03 S at pH 11.0. The specific activity was $9.0 \times 10^6$ liters per mole second at pH 7.2 and $7.8 \times 10^6$ liters per mole second at pH 11.0.

The rotatory dispersion curves of neutral and basic peroxidase are shown in Fig. 5. A Cotton effect was indeed observed at pH 7.2, and the inflection point corresponded very nearly with the absorption peak at 640 μm. The Cotton effect vanished at
pH 11.0. The rotatory dispersion curves of catalase and peroxidase and their changes with pH are, therefore, completely analogous.

It may be concluded that a hemoprotein exhibits a Cotton effect because of the induced asymmetry of the heme by the ordered structure of the protein. The Cotton effect is destroyed when the ordered structure of the protein is destroyed.

Such a generalization is useful in studies of the heme-protein relationship during the denaturation of hemoproteins. An application of this phenomenon is described below.

**Stability of Heme Site in Catalase**—The absorption spectra of catalase, hematin, and “recombined catalase” obtained by the readdition of hematin to apocatalase are compared in Fig. 6. It appears from the similarity of the spectra of native and “recombined” catalase that the heme-protein bonds have been reformed in the latter, although the differences in the ultraviolet part of the spectrum point to changes in the structure of the molecule.

Rotatory dispersion curves were obtained in order to evaluate the nature of these changes.

The dispersion curves for apocatalase and for catalase “reconstituted” at pH 8.5 and 7.5 are reproduced in Fig. 7. All three curves exhibit “plain” dispersion. The apocatalase curve could hardly be expected to behave differently, since the protein has no visible absorption. The “reconstituted” catalase curves, however, are subject to interpretation by our generalization, which leads to the conclusion that the ordered structure of the protein in native catalase is no longer present in the “reconstituted” molecule. A comparison of the rotation curves for native and pH 8.5-reconstituted catalase in Fig. 8 makes a striking case for such a conclusion. Neither apocatalase nor “reconstituted catalase,” incidentally, shows any measurable enzyme activity at the concentrations studied.

The results of a spectrophotometric titration of apocatalase with hematin are given in Fig. 9. It is apparent that the reaction involves 4 moles of heme per mole of the original catalase, or 1 mole of heme per mole of apocatalase, if the latter consists of only one of the four polypeptide chains of the catalase. Such

The titration is analogous to the titration of globin carried out by Rossi-Fanelli, Antonini, and Caputo (23), and the titration curves look very similar.
a stoichiometric reaction, which can be reproduced with samples of apocatalase obtained by different preparative techniques, indicates that the sites on the protein to which the hemins are attached have remained intact throughout the procedures for the removal and subsequent replacement of the heme.

Thus, the rotation experiments reveal that the reconstituted catalase was denatured, whereas the spectrophotometric titration indicates that the heme sites probably remained intact. It therefore appears that, under the conditions of these operations, the structure of the heme site is more stable than the structure of the protein as a whole.

It has been shown previously (23) that globin will react with a stoichiometric quantity of protoporphyrin from which the iron has been removed. This finding demonstrated that the heme sites of the globin have the proper dimensions to accommodate the porphyrin without the involvement of coordinate bond formation with the iron atom. The hemoglobin molecule is therefore remarkably well adapted to fit the porphyrins attached to it. The present investigation points to such a tailor-made heme site for catalase as well.

**SUMMARY**

The rotatory dispersion curve of catalase in neutral solution exhibits a Cotton effect with a peak at 600 mμ and a trough at 635 mμ. A neutral peroxidase solution yields a similar "anomalous" dispersion curve with a 625 mμ peak and a 675 mμ trough. The Cotton effect disappears in catalase solutions at pH 2.8 and pH 11 in a peroxidase solution at pH 11. These results lead to the generalization that the Cotton effect in hemoproteins is correlated with the ordered structure of the protein and vanishes when the structure of the protein is destroyed.

The reaction of apocatalase with hemin is stoichiometric, but the "reconstituted catalase" solution shows no Cotton effect. It is concluded that the structure of the site of heme attachment in catalase is more stable than that of the protein as a whole.

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