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Fine Structure in the Low Temperature Spectra of Catalase Complexes

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Application of rapid freezing and low temperature examination of the absorption bands of catalase Complex I reveals fine structure that is not explained by the presence of Complex II. Such fine structure predominates the region 500–600 mμ, and is not observed in the region of the Soret band.

Knowledge of the chemical nature of the enzyme-substrate compounds of hemoproteins depends critically upon the accuracy of the available physical data. Absorption spectra represent the most precise data[1–3] and any improvement in the accuracy of recording the spectra of these intermediates is of general interest. Here we have achieved an improvement in precision and resolution by rapid freezing of the intermediates and recording of the spectra representing differences between the enzyme-substrate intermediate and the free enzyme.

EXPERIMENTAL

Technique. A particular advantage of observing spectra at liquid nitrogen temperatures[4] is that the proportions of the chemical species are constant over the recording interval. A second advantage is that the recording interval may be as prolonged as desired. A third advantage is that the hemoprotein bands are more distinct at the low temperatures. Thus we have adopted the trapped steady state method, employed for scanning the spectra of the frozen steady state of the cytochromes of the respiratory chain[4–6]. The technique has been improved somewhat over that used previously by increasing the heat capacity of the cuvette (changing aluminium to copper) and by decreasing the volume to 0.25 ml. Thus freezing occurs upon contact between the injected intermediate and the pre-cooled chamber. The center of the chamber cools at a rate of 40° K/sec, as indicated in Fig. 1; it should be noted, however, that the injection of water at 300° K into the chilled cuvette gives a temperature rise approaching the freezing point and rapid cooling occurs thereafter. It is probable that the drop to the freezing point occurs very rapidly in the catalase samples. Experiments with glycerol coating for the cuvette seemed to increase the cooling time only slightly; most of the cooling was from the heat capacity of the copper block itself.

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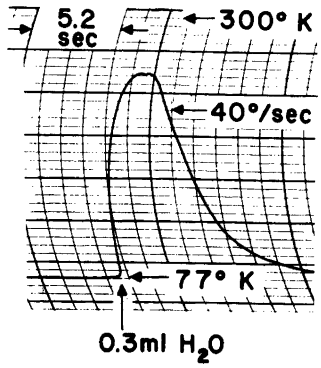

Fig. 1. Thermocouple recording of the time course of cooling of a 0.3 ml liquid sample in the chilled cuvette of the spectroscopic apparatus.

The quick freezing method of Bray[7] has been employed in preliminary tests, but the packing of the frozen material in the cuvettes is not yet sufficiently uniform for precise differential spectrophotometry. Thus we have preferred to use the rigid geometry obtained by rapid freezing in a chilled cuvette. However, further development of this interesting method for precision spectrophotometry is well worthwhile.

Preparations of enzymes and enzyme-substrate compounds. Catalase in the range of 1–4 μM was employed in these experiments in a 2 mm optical path cuvette. The R.Z. values for the three preparations used are 0.77, 1.2, and 1.3 for beef liver (Boehringer), *Micrococcus lysodeicticus* and *Rhodopseudomonas spheroides* catalase. The latter was donated by Dr. R. Clayton. Preparations were diluted in 0.1 M phosphate buffer, pH 6, at 26°C. Ethyl hydrogen peroxide (600 μM) or methyl hydrogen peroxide (60 μM) was used to form the Complex I in less than 1 sec[8,9]. The secondary complex was formed by two successive additions of 600 μM methyl hydrogen peroxide in a 10 min interval[7,8].

The conditions for obtaining complete conversion of catalase to Complex I inevitably led to the formation of small concentrations of Complex II[8,9]. Thus the comparison of the properties of Complex I and II depends upon a comparison of the intensity and position of the bands under conditions which favor maximal conversion of the enzyme to I or II. This procedure was employed in the figures that follow. It is also apparent that the amount of Complex II in our preparation of Complex I was smaller than in Brill and Williams' [10], who employed 6.32 μM catalase and 295 μM ethyl hydrogen peroxide, but required a longer time for measurement.

Procedure. A portion of the diluted catalase solution was injected into the reference portion of the pre-cooled cuvette. The sample was then immersed in liquid nitrogen. After the sample came to thermal equilibrium the cuvette was raised slightly above the surface of the liquid. An appropriate concentration of substrate (between 60 and 600 μM) was blown into the remainder of the catalase solution and the mixture was rapidly sucked up into a 0.5 ml syringe and discharged via a polyethylene catheter into the chilled cuvette. Immediately after filling, the cuvette was plunged beneath the surface of the liquid nitrogen.

Difference spectra were then plotted by a split-beam spectrophotometer covering full scale span ranging from 10 per cent transmission to 0.5 in optical density[11,12]. The original data were replotted against a baseline obtained with catalase in both cuvettes. However, in this paper our attention has been focused upon the location of the peaks of the difference spectra rather than the isosbestic points.

It should be noted that two sensitivities are used in recording the spectra; the vertical dividing line indicates the wavelength at which the sensitivity is altered (usually 470–480 mμ).

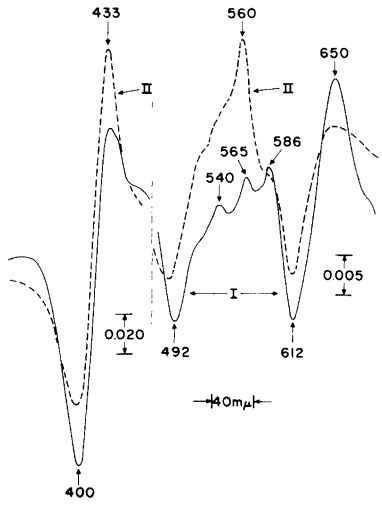

Fig. 2. Difference spectra of catalase complexes under conditions where Complex I (I) or Complex II (II) predominates. 4.7 μM beef liver catalase, 630 μM C_2H_5OOH (I), 1200 μM CH_3OOH (II) (Expt. 775a-2,5).

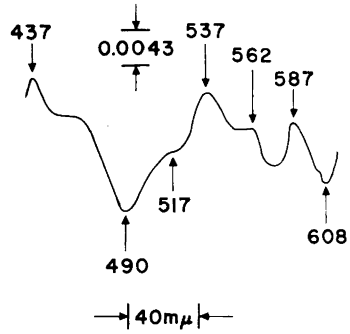

Fig. 3. Difference spectrum similar to Fig. 2, but with 60 μM CH_3OOH (Expt. 775a-3).

RESULTS

Beef liver catalase. Fig. 2 illustrates two low temperature difference spectra obtained at conditions favoring the formation of the primary complex (trace I) or the secondary complex (trace II). Under both conditions the absorption bands of free catalase at 400, 485, and 612 mμ disappear. This corresponds closely to the positions of three of the four absorption bands at 403, 500, 540, and 629 mμ at 300° K[13]. The most prominent band that appears on addition of the low concentration of ethyl hydrogen peroxide is the 650 mμ band. In addition there is a triplet of bands at 540, 566, and 586 mμ which may be considered a part of the Complex I spectrum. A band at 435 mμ, obtained under conditions in which Complex I predominated, was nevertheless difficult to distinguish from that of Complex II, obtained at the higher concentrations of peroxide. However, before considering Complex II, Fig. 3 indicates in more detail the fine structure of the absorption bands obtained at a low concentration of methyl hydrogen peroxide (60 μM or 12 equivalents). Here one can identify distinctive troughs at 490 and 608 mμ and peaks at 437, 537, 562, and 587 mμ. It is of interest that the trough at 608 mμ is not nearly as distinct where a larger conversion of catalase to the intermediate form is observed (Fig. 2).

Considering now the absorption bands of the secondary complex in Fig. 2, it is seen that the predominating bands are at 433 and 560 mμ. In fact, the latter so dominates the "fine structure region" observed at low concentrations of peroxide that they can only be seen as shoulders on the larger band. We would

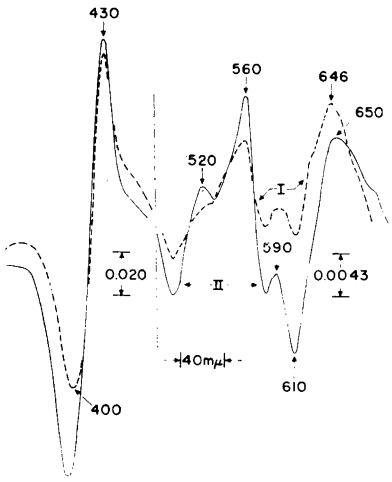

Fig. 4. Difference spectra of catalase complexes as in Fig 2, but using 1.6 μM *M. lysodeicticus* catalase, 120 μM CH_3OOH (I), and 1200 μM CH_3OOH (II) (Expt. 775b-2,3).

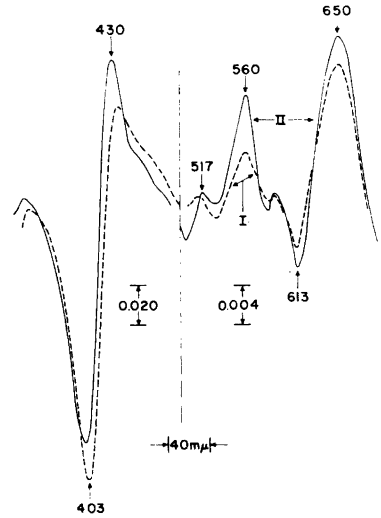

Fig. 5. Difference spectra of catalase complexes as in Fig. 2, but using 1.6 μM *Rhodopseudomonas spheroides* catalase and 60 μM CH_3OOH (I) and 1200 μM CH_3OOH (II) (Expt. 775c-2,3).

therefore tend to identify the band at 650 mμ and the fine structure bands (except that at 560 mμ) with the primary intermediate.

Catalase from M. lysodeicticus. Fig. 4 shows the absorbancy changes resulting on addition of 120 μM methyl hydrogen peroxide; a characteristic absorption band at 650 mμ and the triplet of bands in the region 500–590 mμ again was observed. On repeating the experiment with two additions of 600 μM methyl hydrogen peroxide, the principal increase in intensity is in the bands at 430 and 560 mμ; a much smaller change was observed in the bands at 517, 590, and 650 mμ. Here we associate the bands at 517 and 590 mμ with the primary complex.

Catalase from Rhodopseudomonas spheroides. Fig. 5 shows absorbancy changes due to the addition of low (60 μM) and high (1200 μM) methyl hydrogen peroxide to 1.6 μM *R. spheroides* catalase. The results are similar to those obtained with the other catalases except that the addition of the lower concentration of methyl hydrogen peroxide has led to less than complete development of the 650 mμ band of Complex I. Nevertheless, the triplet of bands shows clearly in the region of 500–600 mμ and the 590 mμ band is not greatly increased at the higher concentration of peroxide; in this case, however, the band at 519 mμ is somewhat more intense at the higher concentration of peroxide.

In Table I we have summarized the band positions obtained at low and high peroxide concentrations for the three types of catalases and it is seen that there is considerable regularity in the positions of all the bands.

Recordings in the ultraviolet region. By inserting a Wratten 39A filter in the optical system, it has been possible to record relatively accurately to 350 mμ.

Table 1. Low temperature spectra of catalase complexes (Expt. 175).

Source of catalase	[E] μM	[S] μM	Type	Test	Predominating complex	Sign of changes +	−	+	+	+	+	−
Beef liver	4.7	630	EtOOH	2a	I	652	610	585	566	540	435	400
	4.7	60	MeOOH	3	I	646	608	587	562	537	437	400
	4.7	1200	MeOOH	5	II	652	610	587	560		433	400
M. lysodeicticus	1.6	600	EtOOH	2	I	650	613	590	560	517	431	399
	1.6	120	MeOOH	3	I	651	610	590	560	516	433	403
	1.6	1200	MeOOH	5	II	652	610		561		430	
R. spheroides	1.6	120	MeOOH	1	I	650	611	592	559	519	431	404
	1.6	1200	MeOOH	3	II	650	610	590	560	517	430	403

In no case were we able to observe an absorption band in this region. It will be noted in Figs. 2, 4, and 5 that the absorption at the shortest wavelengths scarcely rises to the values of absorption above 440 mμ. In short, there seems to be no distinctive absorption in this region comparable to the 650 mμ absorption band.

DISCUSSION

Techniques for the study of labile intermediates. The discovery of the labile primary green intermediates of catalase and peroxide and their subsequent study have been marked by attempts to obtain more and more accurate data. The first description of the difference spectrum of this compound, a point by point evaluation of absorbancy differences between the free enzyme and the labile intermediate (catalase H_2O_2-I), was made at various wavelengths by means of a rapid flow apparatus attached to a double grating monochromator[1]. The absorbancy differences measured at wavelengths in the region 370–450 mμ were recorded and applied to the catalase spectrum. The rapid flow apparatus also has been used to determine the spectra of the primary compounds of catalase and peroxidase with alkyl hydrogen peroxide[2,3]. These methods have attained their ultimate development by the combination of a wavelength scanning spectrophotometer with the rapid flow apparatus[14], which allows the scanning of a difference spectrum over a span of 60 mμ within 9 m sec after mixing. This is, of course, the optimal method for room temperature studies but has a rather large catalase requirement.

Thus the quick freezing method finds a clear application for the accurate examination of spectra of enzymes available in insufficient amounts for the combined flow apparatus and scanning spectrophotometer.

Commercially available instruments have also been used for recording the spectra of these stabilized intermediates of catalase and peroxide; Brill and Williams[10] have employed the Cary spectrophotometer for studies of the spectrum of the primary ethyl hydrogen peroxide complex of bacterial catalase. Even

though they employed the early "single point" method, the recording could not be started until about 10 sec after the addition of the substrate.

Structure of Complex I. Deep interest in the nature of the absorbancy changes accompanying the formation of catalase Complex I arises from the hypothesis presented some time ago[2]: that ring oxidation occurs and that the disappearance of the bands is due to a verdo-hemin structure. This suggestion has been examined in detail by Brill and Williams, who identify the hypothesis by the configuration "ROX". Although their proposal accounts for the retention of the elements of peroxide in the primary complex[15,16], no direct evidence has been presented to date which would permit identification of Complex I as a tautomeric mixture of ROX and POR. It is interesting to note that Brill and Williams have attempted to break down the spectrum of Complex I into three components, having maxima at 340–360, 405–415, and 450–460 mμ. Since it is obvious that these three components, if they exist at all, are inadequately resolved at room temperature, it occurred to us that they might be adequately resolved at low temperatures. The experimental results appear to be strongly against the possibility that low temperatures would resolve these three components of Complex I. Since the low temperatures do show a variety of characteristic bands of hemoproteins in the visible region, it is likely that fine structure in the region 350–450 mμ would have been revealed by this method, if it existed.

If, indeed, catalase Complex I is a mixture of intermediates of different values of spin, the data presented here indicate that the visible region of the spectrum (500–600 mμ) is a much preferable region in which to attempt kinetic or other studies to identify their separate existences.

The existence of the fine structure in the spectra of the primary catalase complexes is of especial interest in view of the increasing evidence that they consist of a peroxide complex of trivalent iron[15,16].

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