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Catalase of *Neurospora crassa*. 2. Electron Paramagnetic Resonance and Chemical Properties of the Prosthetic Group†

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ABSTRACT: The inducible catalase from *Neurospora crassa* 5297a when examined at 13 K exhibits electron paramagnetic resonance (9 and 34 GHz) with two sets of principal features at $g = 6.33$, 5.48, and 1.99 and 6.62, 5.18, and 1.99. Minor amounts of other resonances are seen near $g = 6$. Sodium formate converts these to a single species with features at $g = 6.51$, 5.34, and 1.99. This is consistent with the presence of two or more high-spin ferric porphyrin complexes in the untreated enzyme. The azide complex of this catalase yielded a single well-defined set of resonances at $g = 2.50$, 2.26, and 1.87, indicating that the high-spin forms of the enzyme are converted to a single low-spin compound under these circumstances. Integration of this signal indicated that at least 90% of the heme iron in the native enzyme is present as high-spin ferric complexes. In some preparations of the untreated enzyme small amplitude resonances at $g = 2.42$, 2.30, and 1.89 were observed; these appear to arise from inactive forms of the enzyme. Extraction of the heme group and subsequent removal of iron and conversion to the methyl ester yield a porphin with absorbance maxima at 653, 599, 533, 500, and 399 nm in a ratio of 2.92:0.33:0.33:1.00:12.9. The spectrum closely resembles that of the chlorin prepared by J. Barrett [(1956) *Biochem. J. 64*, 626] from cytochrome a_2 of *Escherichia coli* and is distinct from any reported porphyrin spectrum. The chromatographic properties of the catalase chlorin suggest the presence of approximately four carboxyl groups, whereas chlorin a_2 is reported to have two carboxyl groups. We were unable to reoxidize the presumed chlorin to a porphyrin by using 2,3-dichloro-5,6-dicyanobenzoquinone, a phenomenon observed previously with sterically hindered chlorins and phorbins [Woodward, R. B. (1961) *Pure Appl. Chem. 2*, 383]. Nonetheless, the evidence presented strongly suggests that, unlike previously studied catalases, the inducible catalase of *N. crassa* has as its prosthetic group a polar high-spin ferric dihydroporphyrin complex.

Catalase, the enzyme that converts hydrogen peroxide to oxygen and water, was first shown by Stern (1936) to contain protohematin as the prosthetic group of the enzyme. Since then, catalases from every organism studied have been found to contain this type of heme. Included are the enzymes from a diverse number of sources such as mammalian liver and erythrocytes (Stern, 1936; Herbert & Pinsent, 1948b; Bonnichsen, 1947; Nagahisa, 1962; Higashi et al., 1966), bacteria (Herbert & Pinsent, 1948a; Clayton, 1959), and yeast (Seah & Kaplan, 1973; Fujii & Tonomura, 1975). Catalases are known to be comprised of four subunits (Tanford & Lovrien, 1962; Schroeder et al., 1969; Kiselev et al., 1968) and contain four hematin prosthetic groups per molecule. Except for the enzyme from yeast, all catalases have molecular weights in the range of 225 000–270 000.

In the first paper of this series we reported a catalase isolated from *Neurospora crassa* that is different from other catalases in that the molecular weight is higher (320 000) and the enzyme contains a novel heme. These last results were based on spectrophotometric analyses of the native enzyme and of the pyridine hemochrome derived from it. The native enzyme is green in color and exhibits two absorption bands with maxima at 400 and 590 nm ($A_{400}/A_{590} = 4.89$). The spectrum of the pyridine hemochrome was also found to be unique. The

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enzyme was shown to contain 3–4 iron atoms/mol. We report here further studies on the nature of the prosthetic group and its involvement in the enzyme.

Materials and Methods

Materials. Spectrophotometric grade benzene and chloroform were purchased from Fisher Scientific, protohemin and protoporphyrin IX dimethyl ester from Sigma, and silicic acid (Silicar TLC-7G) and spectrophotometric grade pyridine from Mallinckrodt. The reagents for polyacrylamide gel electrophoresis were purchased from Eastman Kodak. Chlorophylls *a* and *b* were isolated from fresh spinach leaves according to Strain et al. (1963). Pheophytins *a* and *b* were made from the corresponding chlorophylls according to Smith & Benitez (1955). All other chemicals were analytical reagent grade and used as supplied.

Spectroscopic Measurements. EPR[1] spectra were recorded on 0.25-mL samples contained in 4-mm (i.d.) quartz tubes at 13 K with a Varian Model E-9 spectrometer. The temperature was controlled by a stream of helium boil-off gas (Tsai et al., 1970). For quantitation of the heme signal, a 250-μL sample of 43 μM catalase was mixed with 50 μL of 55 mM NaN_3 and allowed to stand at 25 °C for 5 min, after which it was frozen and the spectrum recorded again. Addition of azide resulted in conversion of essentially all of the high-spin ferric heme to a low-spin form with a more easily integratable spectrum. A double integration was performed on the sample as well as on a standard solution of cupric EDTA complex in water (Aasa & Vänngard, 1975). The double integration was corrected for the small fraction of high-spin heme not converted to the low-spin form.

The 34-GHz spectra were performed on a modified Varian V-4500 spectrometer. The cooling to 13 K was provided by helium boil-off gas flowing through a Dewar enclosing the cavity. The samples were contained in 1-mm (i.d.) quartz tubes.

Fluorescence spectra (excitation and emission) were determined with an Aminco-Bowman spectrofluorometer. Absorption spectra were measured at room temperature in 1-cm path length cuvettes with a Cary Model 14 recording spectrophotometer equipped with 0–0.1 and 0–1 optical density slide-wires.

Extraction and Chemical Modification of N. crassa Catalase Heme. N. crassa catalase was prepared as described previously (Jacob & Orme-Johnson, 1979). The heme prosthetic group was extracted by the method of Murphy et al. (1973). To 1 vol of a solution of *N. crassa* catalase in buffer A[2] was added 9 vol of ice-cold acetone–0.015 N HCl. After the solution was allowed to stand for 5 min at 0 °C, it was centrifuged and the supernatant decanted. Pyridine was added to the supernatant to a final concentration of 10%. Since TLC analysis revealed only one type of prosthetic group present in the catalase, a chromatographic step was not required after the extraction. The heme was dried in a stream of N_2 and stored in a desiccator at −15 °C.

The iron was removed from the *N. crassa* heme by the method of Murphy et al. (1973).

The porphin methyl ester was made by either of the two methods described by Murphy & Siegel (1973). After esterification, porphin methyl esters were extracted with benzene and purified by thin-layer chromatography in silicic acid by using a benzene–ethyl acetate–methanol–butanol (82:14:3:1) solvent system (Murphy et al., 1973). The porphin ester was located by its red fluorescence and recovered by elution with ether. Contaminating silicic acid was subsequently removed by centrifugation.

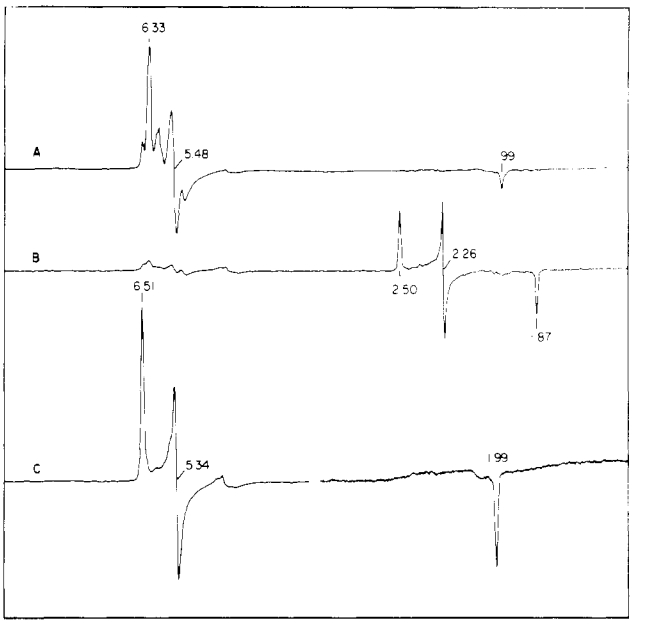

FIGURE 1: EPR spectra of *N. crassa* catalase. (A) Solution of 43 μM enzyme as isolated; (B) sample from part A treated with NaN_3 as explained in Materials and Methods; (C) solution of 18.5 μM native enzyme in 0.2 M sodium formate. Conditions: microwave frequency, 9.19 GHz; microwave power, 10 mW; modulation frequency, 100 kHz; modulation field, 10 G; sweep rate, 1000 G/min; time constant, 0.1 s; temperature, 13 K. The magnetic strength increases linearly from left to right; the ordinate is an arbitrary function of the first derivative of microwave absorption. Some values of $g = \nu/\beta H = 0.71445\nu/H$, where ν is the microwave frequency in megahertz and H is the magnetic field strength in gauss, are presented in the figure. Relative instrumental gain used for parts A, B, C-left, and C-right was 1:1.56:1.56:6.24.

Analytical Methods. Iron was determined by the method of Van De Bogart & Beinert (1967). Analytical polyacrylamide gel electrophoresis was performed by the procedure of Jacob & Orme-Johnson (1979).

Results

EPR Spectra. We believe for reasons given below that the 9-GHz spectrum of homogeneous *N. crassa* catalase, shown in Figure 1, is primarily composed of two overlapping sets of rhombic signals having resonances at g = 6.33, 5.48, and 1.99 and 6.62, 5.18, and 1.99. Rhombic signals of this type are characteristic of high-spin ferric heme. [Electronic spectra of the enzyme and its pyridine hemochrome [cf. Jacob & Orme-Johnson (1979)] leave little doubt about the presence of heme.] In addition to the major resonances indicated above, some enzyme preparations were found to give other minor EPR resonances in the g = 6 region, but these were found to vary in intensity from preparation to preparation.

Also shown in Figure 1 is the spectrum of the native enzyme in the presence of 9 mM sodium azide. The complex spectrum is converted to a single rhombic low-spin pattern with resonances at g = 2.50, 2.26, and 1.87. This is in contrast to the report by Williams-Smith & Patel (1975) that the azide complex of bovine liver catalase exists as the high-spin form. Furthermore, as shown in Figure 1 (bottom) native enzyme treated with 0.2 M sodium formate (pH 7) is converted to a single rhombic high-spin species with resonances at g = 6.51,

[1] Abbreviations used: EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; TLC, thin-layer chromatography.

[2] Buffer A = 0.1 M potassium phosphate, pH 7.3, 10^{-3} M phenylmethanesulfonyl fluoride, 10^{-3} M dithiothreitol, and 5×10^{-4} M EDTA.

5.34, and 1.99. The presence of one rhombic signal for either low-spin ferric heme–azide or high-spin ferric heme–formate suggests that the native enzyme contains only one type of heme group. We presume that the small signal observed at $g = 4.3$ is due to adventitious nonheme iron.

A double integration performed on the spectrum of the low-spin azide complex, as described under Materials and Methods, indicated the presence of 3.0 mol of heme/mol of enzyme. This was in reasonable agreement with the value of 3.4 obtained for iron by the method of Van De Bogart & Beinert (1967).

Figure 2 shows an EPR spectrum of catalase at 34 GHz. The same type of line shape was observed as at X-band frequency. However, increased resolution at the higher frequency resulted in the detection of additional resonances not observed in the 9-GHz spectrum. Computed g values for the major resonances at the two frequencies are virtually identical and indicate that the complexity of the spectrum is not a consequence of nuclear hyperfine interactions, but rather represents magnetically inequivalent paramagnetic species. Thus, we believe that the spectrum consists of nested sets of rhombic signals centered at $g = 6$, as suggested above.

A power study of the $g = 6$ region indicated no saturation over the power range 0.1–10 mW at 13 K. Appreciable broadening of the signals at 10 mW did occur above 60 K. In addition, the resonances with g values at 6.62 and 5.18 were found to broaden at a lower temperature than the less rhombically distorted pair at $g = 6.33$ and 5.48, supporting the suggestion that they are respective features of two distinct signals.

Comparison of EPR Spectra of Different Enzyme Preparations. Throughout this study various preparations of *N. crassa* catalase gave somewhat different EPR spectra. Two types of differences were observed. (1) There was variation in the amount of a low-spin ferric heme signal[3] at $g = 2.42$, 2.30, and 1.89. This signal was large in lower specific activity material and thus seemed to be correlated with inactive material which could not be removed by the steps employed in the purification. (2) There were differences in the number and ratio of signals in the $g = 6$ region. These differences were not correlated with any variation in specific activity, and these species represent catalytically active high-spin variants of the enzyme.

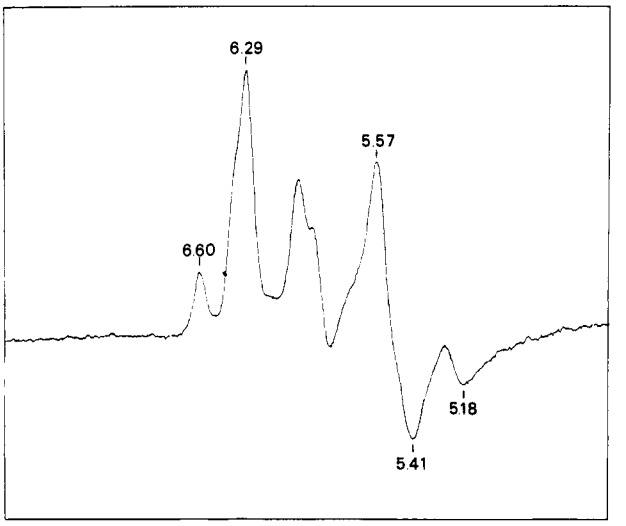

FIGURE 2: EPR spectrum of *N. crassa* catalase (125 μM) at 34-GHz microwave frequency. Conditions of EPR spectroscopy: microwave power, 5.5 mW; modulation frequency, 100 kHz; modulation field, 5 G; sweep rate, 1600 G/min; time constant, 0.5 s; temperature, 13 K. The magnetic strength increases linearly from left to right; the ordinate is an arbitrary function of the first derivative of microwave absorption. Some values of $g = \nu/\beta H = 0.71445\nu/H$, where ν is the microwave frequency in megahertz and H is the magnetic field strength in gauss, are presented in the figure.

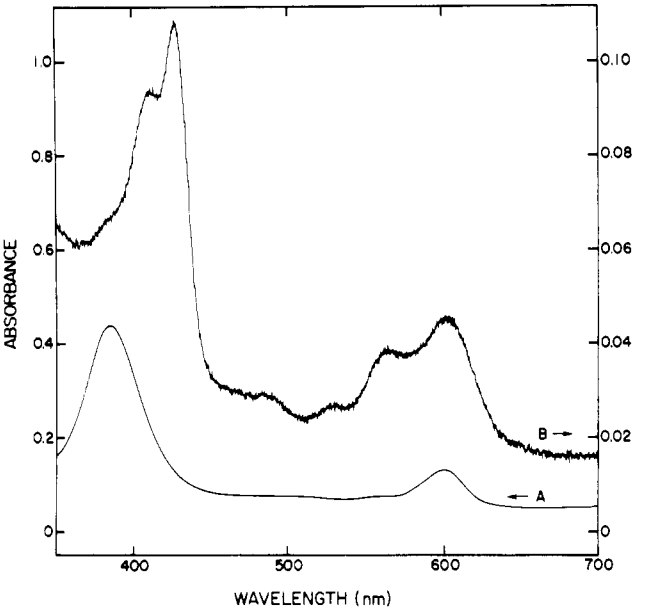

FIGURE 3: Electronic spectra of extracted heme from *N. crassa* catalase. (A) Extracted heme in acetone–HCl was prepared as described in Materials and Methods and immediately recorded vs. a blank containing one part of buffer A and nine parts of 0.015 N HCl in acetone. (B) Extracted heme in pyridine was recorded vs. a pyridine blank. The heme–pyridine complex was prepared as described in Materials and Methods.

Characterization of the Prosthetic Group. A. Properties of the Iron–Porphin Complex. The heme was isolated by acid–acetone extraction (Murphy et al., 1973). An absorption spectrum of the acetone–HCl supernatant after centrifugation is shown in Figure 3. The spectrum exhibits two peaks, one at 387 nm and the other at 602 nm, $A_{387}/A_{602} = 5.18$. Pyridine, when added to a final volume of 10%, resulted in

[3] As noted in Results, some preparations of enzyme isolated by the method of Jacob & Orme-Johnson (1979) were found to contain a low-spin heme signal as well. They also had lower specific activities. The low-spin heme signal is almost certainly attributable to denatured catalase rather than to a separate heme protein type on the basis of the following observations. (1) A preparation containing substantial amounts of low-spin heme was subjected to polyacrylamide gel electrophoresis. It exhibited only one heme band. (2) Heme extracted by acid–acetone from a sample containing a high percentage of low-spin heme exhibits exactly the same spectrum as a sample obtained from a preparation devoid of the low-spin form. (3) Preparations containing the low-spin heme, when rechromatographed on Sephadex G-200, exhibited a single symmetrical peak of protein having a constant but lower than normal purity index. (4) The higher the content of low-spin heme, the lower the specific activity of the catalase. (5) An increase in the amount of low-spin heme at the expense of the high-spin species was observed in one preparation during the later stages of purification. (6) The g values for the low-spin heme signal of native enzyme did not coincide with those for the low-spin form produced by incubation in azide or cyanide, and the low-spin heme contained in native enzyme preparations was *not* convertible to the low-spin azide form. The low-spin signal of *N. crassa* catalase must therefore be due to a ligand which is either an amino acid residue of the protein or a nonexchangeable small molecule. Whatever the identity of the sixth ligand, either it is tightly bound so that azide cannot exchange with it during the time (minutes) we allowed or a conformational change has occurred obstructing access of solvent to the heme group. The latter interpretation is attractive since the heme groups of catalases are thought to be deeply buried in the interior of the molecule (Deisseroth & Dounce, 1970). The enzymatic rate of bovine liver catalase drops off rapidly as increasingly bulky alkyl peroxides are substituted for peroxide, indicative of limited access of bulky molecules to the prosthetic groups (Chance, 1950). A small conformational change might entirely obstruct access of solvent to the heme.

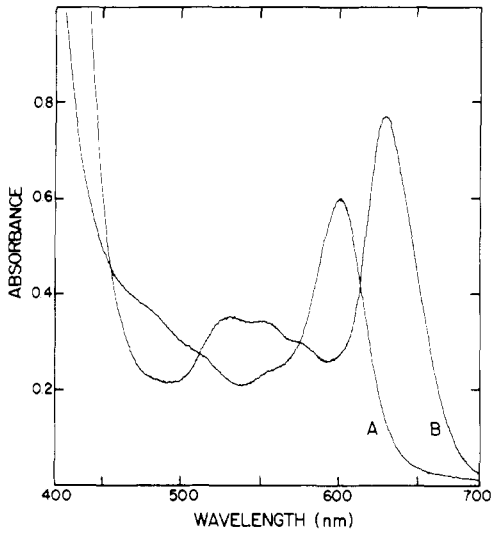

FIGURE 4: Demetalization of the *N. crassa* catalase heme. (A) *N. crassa* catalase heme, ~20 nmol, was dissolved in 1 mL of glacial acetic acid, and the spectrum was immediately recorded vs. a glacial acetic acid blank. (B) *N. crassa* catalase heme in 1 mL of glacial acetic acid under a stream of N_2 and at room temperature was treated with 25 μL of 1% $FeSO_4$ in concentrated HCl and stirred for 1 min. The spectrum of the solution was immediately recorded vs. a blank containing 39 parts glacial acetic acid and one part 1% $FeSO_4$ in concentrated HCl.

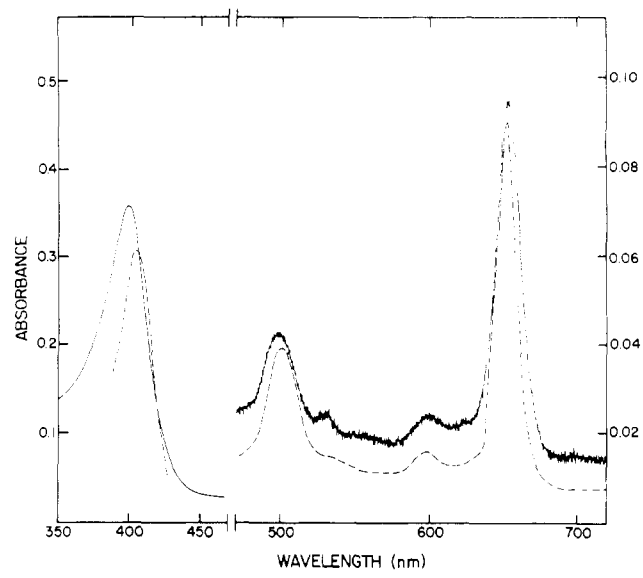

FIGURE 5: Electronic spectra of *N. crassa* catalase porphin methyl ester and chlorin a_2 in ether. The porphin methyl ester (—) was prepared and purified as described in the text. The chlorin a_2 spectrum (- - -) was a replot of the spectrum reported by Barrett (1956).

the formation of a precipitate. After a second centrifugation, the green supernatant was brought to near dryness in a stream of N_2 gas and diluted with pyridine. A spectrum of the resultant pyridine complex is shown in Figure 3. This spectrum was identical with the pyridine hemochromogen spectrum of the catalase (Jacob & Orme-Johnson, 1979) except for additional detail below 430 nm due to the elimination of absorption by dithionite and protein in the purified sample. Maxima (relative absorbance ratios in parentheses) were found at 412 (7.33), 429 (8.76), 483 (1.19), 528 (1.00), 565 (2.14), and 602 (2.76) nm.

Thin-layer chromatography of the extracted heme was performed in a solvent system composed of 2,6-lutidine–water (5:3) (NH_3 vapor). The chromophore ran as a single green spot exhibiting some tailing and having an R_f of 0.89. An authentic sample of protohemin, chromatographed simultaneously, gave an R_f of 0.49. This behavior, in addition to the spectrophotometric differences between the two substances, indicated that the catalase chromophore was *not* protohemin.

B. Properties of the Porphin. Demetalization of the heme was performed by a procedure referred to in Materials and Methods. In Figure 4 the absorption spectrum of the chromophore in glacial acetic acid immediately before and after the iron removal step is shown. An accurate determination of the Soret maximum could not be made due to the rising absorbance below 400 nm. Maxima were found at 600 nm for the heme and at 629 (2.7), 576 (1.0), 552 (1.2), and 530 (1.2) nm for the same solution after the iron removal step. The resultant solution was found to be fluorescent, indicating that iron removal had occurred. The porphin was brought to dryness in a stream of N_2 and stored in the same manner as the heme. The fluorescence spectrum of the porphin in 1.5 N HCl was obtained. Excitation of the sample at 410 nm gave rise to one emission peak at 627 nm. The excitation spectrum of the porphin acid was similar to its absorption spectrum in 1.5 N HCl (not shown) and glacial acetic acid (Figure 4). The porphin was found to be insoluble in benzene.

C. Properties of the Porphin Methyl Ester. The porphin methyl ester was made by either of the two methods referred to in Materials and Methods. The absorption spectrum of the porphin methyl ester formed in methanol–H_2SO_4 after 4 days of incubation in this solvent at −10 °C gave wavelength maxima at 632 (1.90), 578 (0.80), 550 (1.00), 536 (1.03), 398 (19.0), and 365 (16.5) nm. After esterification by this method, the porphin ester was extracted with benzene and found to have a spectrum identical with that of a sample of porphin methyl ester prepared with diazomethane and extracted and purified by the method of Murphy et al. (1973). In Figure 5 is the absorption spectrum of the neutral porphin methyl ester in ether after purification by thin-layer chromatography. It had absorbance maxima at 653 (2.92), 599 (0.33), 533 (0.33), 500 (1.00), and 399 (12.9) nm. The spectrum of the porphin methyl ester in chloroform or dioxane was virtually identical with the spectrum in ether (1–2-nm shifts in the principal maxima were observed). The spectrum was strikingly similar to that of a chlorin (dihydroporphyrin); moreover, the A_{Soret}/A_{α} of the porphin methyl ester in ether was 4.4, a typical value for a chlorin structure, as opposed to ~50 for porphyrins (Falk, 1964).

The fluorescence spectrum of the porphin methyl ester in dioxane was found to give one emission peak at 647 nm by using 405-nm excitation.

The chromatographic behavior of the porphin methyl ester was compared with protoporphyrin dimethyl ester, pheophytin *a*, and pheophytin *b* in a benzene–ethyl acetate–methanol–butanol (82:14:3:1) system. Thin-layer chromatography in this system is thought to separate porphyrin methyl esters on the basis of their methyl ester content. R_f values of 0.61, 0.81, 0.85, and 0.85 were found for *N. crassa* catalase porphin methyl ester, protoporphyrin dimethyl ester, pheophytin *a*, and pheophytin *b*, respectively. Doss (1967) reports values of 0.85, 0.80, and 0.66 for protoporphyrin dimethyl ester, deuteroporphyrin dimethyl ester, and coproporphyrin tetramethyl ester, respectively. The *N. crassa* porphin methyl ester thus migrates with an R_f value near that of coproporphyrin tetramethyl ester, an indication that the prosthetic group contains four carboxyl moieties.

To confirm that the structure was that of a chlorin as well as learn more about its substituents, conversion to the corresponding porphyrin was attempted. 2,3-Dichloro-5,6-dicyanobenzoquinone, known to oxidize simple chlorins to

porphyrins in benzene at room temperature (Eisner et al., 1957), was totally ineffective in altering the absorption spectrum of the porphin methyl ester under conditions normally used for the conversion. Lack of reactivity with this quinone has previously been reported for phorbins and highly substituted chlorins and is thought to be due to steric hindrance by the side chains of these compounds (Woodward, 1961).

A study of the literature of porphins did not result in the identification of the *N. crassa* chromophore; however, the spectrum of chlorin a_2, reported by Barrett (1956), is quite similar to that of *N. crassa* chlorin (Figure 5). The position and relative intensity of the absorption maxima are essentially identical except for a difference of 6 nm in the position of the Soret peak, 405 nm for chlorin a_2 as opposed to 399 nm for the catalase porphin methyl ester. Also, there is a discrepancy in the apparent carboxylic acid residue content. Barrett (1956) reports that chlorin a_2 most likely contains two residues, whereas thin-layer chromatography suggests that catalase chlorin methyl ester probably contains four. Chlorin a_2, although extensively purified, is lipophilic in character, whereas catalase chlorin is quite polar and not soluble in benzene or ether until esterified. Table I (see paragraph concerning supplementary material at the end of this paper) contains spectral data for *N. crassa* catalase porphin methyl ester, chlorin a_2, and a selection of chlorins reported by Fischer & Orth (1940) which resemble the *N. crassa* chlorin with respect to the positions of the absorption bands. None of these spectra appear to be as similar to that of catalase chlorin as that of chlorin a_2, with the possible exception of pyrrochlorin *e*, which, however, contains only one carboxylic acid residue.

Discussion

The objectives of this work were two: to investigate the EPR properties of *N. crassa* catalase and to extract and characterize the prosthetic group of the enzyme.

Homogeneous *N. crassa* catalase is found to exhibit an EPR spectrum consisting predominately of high-spin ferric heme signals. The $g = 6$ region reveals at least two major and a minor pair of g values, each nested pair representing a ferric chlorin of a different symmetry form. Since conversion to the low-spin signal by azide results in only a single rhombic EPR signal, we interpret the $g = 6$ signal complex to be due to perturbation of equivalent ferric chlorin prosthetic groups by their local environments. This perturbation is apparently either relieved by or small compared to the effect of coordination of the strong-field ligand azide. The same arguments can be applied to the rhombic signal produced by the weak-field ligand formate. The following observations suggest that the complexity of the $g = 6$ signal may in part arise during purification. (1) Enzymes from successive preparations exhibit slightly different signals in the $g = 6$ region. (2) Changes in the (composite) line shape of the complex signal are observed between different stages of purification in a given enzyme preparation. Blumberg & Peisach (1971) have reported that commercial preparations of bovine liver catalase contain ferric heme in two different symmetry forms. Preparations could be converted entirely to either form by gel exclusion chromatography or dialysis. Incubation at 0 °C for 3 days reestablished the equilibrium populations of the two symmetry forms, thus indicating that the forms were interconvertible.

More recently, Blum et al. (1978) have shown that interconversion of the two forms can be produced by varying the pH. They suggest that protonation of a histidine side chain exerts a strong influence on the local heme symmetry and is responsible for the interconversion between forms. The minor variations in the $g = 6$ region found for *N. crassa* catalase may simply be the result of slight differences in the pH of the various enzyme preparations.

With the limited amounts of enzyme available, we have thus far sought mainly to classify the prosthetic group by spectroscopic means. The properties of the extracted porphin and the corresponding methyl ester clearly suggest the presence of a dihydroporphyrin (chlorin) nucleus. The chlorin is probably a fairly polar compound since it is insoluble in benzene. Upon thin-layer chromatography in a benzene–ethyl acetate–methanol–butanol (82:14:3:1) solvent system, the esterified chlorin was found to migrate with an R_f value consistent with four methyl ester residues per molecule.

The possibility that the prosthetic group of *N. crassa* catalase was somehow chemically altered by the presence of the protease inhibitor phenylmethanesulfonyl fluoride during the purification was ruled out by an isolation performed in the absence of the inhibitor. However, the yield of enzyme was low (~30% of control values) in the absence of phenylmethanesulfonyl fluoride, an indication that the enzyme is susceptible to proteolytic degradation. Similarly, a preparation performed in the absence of EDTA [cf. Jacob & Orme-Johnson (1979)] gave results identical with those of a preparation performed in its presence, thus ruling out any EDTA involvement.

This appears to be the first example of a *catalase* that does not contain protohematin as its prosthetic group. Myeloperoxidase has been previously shown (Morell & Chang, 1967) to contain a chlorin as its prosthetic group; however, the chlorin is covalently attached to the enzyme and cannot be released by acid–acetone extraction as can the prosthetic group of this catalase. The evidence presented above supports the hypothesis that the prosthetic group of this inducible *N. crassa* catalase is a ferric chlorin, presumably with four carboxylate side chains. The isolated enzyme exists in multiple high-spin forms, and an inactive low-spin form of the enzyme is present in some preparations. The electronic spectrum most closely resembles that of bacterial chlorin a_2.

Acknowledgments

Thanks are due to Professor H. Beinert for the use of the 35-GHz EPR spectrometer and to Professors D. Mauzerall and L. M. Siegel for helpful suggestions concerning the chemistry of porphyrins. We also thank Professor J. Peisach for suggesting that formate binding might be a source of the multiplicity of high-spin species in beef liver catalase, thus prompting us to test this ligand with *N. crassa* catalase.

Supplementary Material Available

Table I containing spectral data for *N. crassa* catalase porphin methyl ester, chlorin a_2, and a selection of chlorins reported by Fischer & Orth (1940) (1 page). Ordering information is given on any current masthead page.

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Nucleophile in the Active Site of *Escherichia coli* Galactose-1-phosphate Uridylyltransferase: Degradation of the Uridylyl-enzyme Intermediate to N^3-Phosphohistidine†

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ABSTRACT: The [^{32}P]uridylyl-enzyme intermediate form of *Escherichia coli* galactose-1-P uridylyltransferase can be converted to a [^{32}P]phosphoryl-enzyme by first cleaving the ribosyl ring with $NaIO_4$ and then heating at pH 10.5 and 50 °C for 1 h. After alkaline hydrolysis of the [^{32}P]phosphoryl-enzyme the major radioactive product is N^3-[^{32}P]-phosphohistidine. A lesser amount of $^{32}P_i$ is also produced as a side product of the hydrolysis of N^3-[^{32}P]phosphohistidine. No N^1-phosphohistidine, N^ϵ-phospholysine, or phosphoarginine can be detected in these hydrolysates. It is concluded that the nucleophile in galactose-1-P uridylyltransferase to which the uridylyl group is bonded in the uridylyl-enzyme intermediate is imidazole N^3 of a histidine residue. This degradation procedure should have general applicability in the degradation and characterization of nucleotidyl-proteins.

Structural characterization of covalently bonded enzyme–substrate intermediates and covalently modified regulated enzymes remains a challenging task in biochemistry. Among the known covalently modified enzymes, only the phosphoryl-enzymes and the aldimine(or ketimine)-enzymes are routinely subject to general degradative procedures for identifying the nature of covalent bonding and the amino acid residue involved.

The nucleotidyl-enzymes which have been discovered and studied over the past 10 years present special degradation problems, some of which are well exemplified by the uridylylgalactose-1-P uridylyltransferase discovered and studied in this laboratory (Wong & Frey, 1974a; Wong et al., 1977). This uridylyl-enzyme is the intermediate in the interconversion of galactose-1-P and UDP-glucose with glucose-1-P and UDP-galactose (Wong & Frey, 1974b; Wong et al., 1977). The uridylyl group is known from the hydrolytic properties of the uridylyl-enzyme to be bonded to a nitrogen atom in the enzyme. On the basis of data from chemical modification with diethyl pyrocarbonate, it appears that this may be one of the nitrogen atoms in the imidazole ring of a histidine residue (Wong et al., 1977). Direct degradation of the intermediate to uridylylhistidine for structural characterization is not presently feasible because the protein is refractory to enzymatic degradation. Moreover, the acid lability of the phosphoramidate linkage and the instability of the uracil ring in strongly alkaline solutions rule out the possibility that the uridylyl-enzyme might be hydrolyzed directly to a uridylyl amino acid.

In this paper we report on the degradation of this uridylyl-enzyme to a phosphoryl-protein and then to N^3-phosphohistidine by a procedure which should have general applicability for characterizing nucleotidyl-proteins when the nucleotidyl group is bonded to serine, threonine, lysine, arginine, or histidine.

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