NADPH binding and control of catalase compound II formation: comparison of bovine, yeast, and *Escherichia coli* enzymes

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1. NADPH binds to bovine catalase and to yeast catalases A and T, but not to *Escherichia coli* catalase HPII. The association was demonstrated using chromatography and fluorimetry. Bound NADPH fluoresces in a similar way to NADPH in solution. 2. Bound NADPH protects bovine and yeast catalases against forming inactive peroxide compound II either via endogenous reductant action or by ferrocyanide reduction during catalytic activity in the presence of slowly generated peroxide. 3. Bound NADPH reduces neither compound I nor compound II of catalase. It apparently reacts with an intermediate formed during the decay of compound I to compound II; this postulated intermediate is an immediate precursor of stable compound II either when the latter is formed by endogenous reductants or when ferrocyanide is used. It represents therefore a new type of hydrogen donor that is not included in the original classification of Keilin and Nicholls [Keilin, D. and Nicholls, P. (1958) Biochim. Biophys. Acta **29**, 302–307] 4. A model for NADPH action is presented in which concerted reduction of the ferryl iron and of a neighbouring protein free radical is responsible for the observed NADPH effects. The roles of migrant radical species in mammalian and yeast catalases are compared with similar events in metmyoglobin and cytochrome *c* peroxidase reactions with peroxides.

INTRODUCTION

The discovery that bovine liver catalase binds NADPH tightly was made unexpectedly by Kirkman and Gaetani (1984) while trying to identify NADP(H)-binding proteins in erythrocytes. Human, canine and bovine catalases bind one dinucleotide molecule per subunit, the binding of the reduced dinucleotide is favoured over the oxidized, and NADPH is the dinucleotide with the lowest K_D (< 10 nM). In the crystallographic structure, a region of extra density previously misinterpreted as a C-terminal extension proved to be the cofactor, which occupies a small cleft between a 'hinge' and a β-barrel domain (Fita and Rossmann, 1985b). These mammalian catalases all bind NADPH (Kirkman et al., 1987), as do the catalases of *Proteus mirabilis* and *Micrococcus lysodeikticus* (Jouve et al., 1989, 1991). Potato catalase does not bind NADPH (Beaumont et al., 1990), and a search for NADPH binding by other plant catalases is awaited. *Aspergillus niger* (Kirkman and Gaetani, 1984) and *Penicillium vitale* (Fita and Rossman, 1985b) catalases do not show NADPH-binding sites. *Penicillium vitale* catalase (Melik-Adamyan et al., 1986) has an additional C-terminal 'flavodoxin' domain (Vainshtein et al., 1986) which involves the same crevice between α-helix and β-barrel domains that comprises the bovine enzyme NADPH-binding site (Melik-Adamyan et al., 1986).

The proximal iron ligand in eukaryotic catalase is Tyr-357 (Reid et al., 1981; Fita and Rossman, 1985a). In other haemoproteins, this position is commonly occupied by histidine: His-170 in horseradish peroxidase (Dunford, 1991), His-175 in cytochrome *c* peroxidase (Bosshard et al., 1991) and His-93 in myoglobin (Takano, 1977). Myoglobin with this proximal group has a characteristic absorption spectrum (Adachi et al., 1991, 1993), and the His93Tyr mutant has a (ferric) spectrum similar to that of catalase. Resonance Raman spectroscopy indicates Fe–tyrosine ligation in mammalian, *A. niger* and *Micrococcus luteus* catalases (Sharma et al., 1989), and in *Escherichia coli* HPII catalase (Dawson et al., 1992). Catalase haem iron is high-spin and probably pentaco-ordinate (Chuang et al., 1988; Sharma et al., 1989). The reaction with peroxide produces a ferryl iron plus porphyrin π-cation radical intermediate structure, known as compound I (Dolphin et al., 1971). This is followed either by a two-electron reduction to the ferric state or by a one-electron reaction yielding the inhibitory peroxide compound II (Nicholls, 1964), in which the iron remains ferryl but the porphyrin radical has been lost.

Two hypotheses for NADPH function have been proposed (Kirkman and Gaetani, 1984). In the first, catalase possesses an NADPH-dependent activity distinct from its catalatic and peroxidatic modes. Although the cellular functions of catalase remain unclear (Oshino and Chance, 1977), the demonstration of a role involving NADPH in catalytic reactions would necessitate a fundamental re-examination of enzyme structure and behaviour [see Percy (1984) and Vuillaume et al. (1988)].

In the second hypothesis, NADPH protects the enzyme from H_2O_2 inactivation. Kirkman et al. (1987) found that NADPH prevents compound II accumulation without reaction with compound I. They proposed that NADPH is a unique one-electron donor, which would reduce compound II back to ferricatalase, and Almarsson et al. (1993) have presented a more elaborate version of this model. Eaton et al. (1972) noted protection of catalase activity by NADPH in haemolysates incubated with ascorbate. In contrast, Orii et al. (1989), who studied perfused rat livers, suggested that compound II does accumulate *in vivo* to appreciable levels.

Yeast catalases are less well characterized, and neither crystallographic structure nor chemically determined amino acid sequences are available for any *Saccharomyces cerevisiae* isoenzyme. Brown (1953) originally isolated catalase from yeast, and Seah and Kaplan (1973) and Seah et al. (1973) purified it to

Abbreviations used: BLC, bovine liver catalase; HP, hydroperoxidase (catalase) of *Escherichia coli*.

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homogeneity. They obtained two fractions by hydroxyapatite fractionation: catalase A ('Atypical'), and catalase T ('Typical'). The latter had a subunit molecular mass of 61 kDa, a native molecular mass of 244 kDa, and an activity similar to those of other catalases. Catalase A had an unusual SDS/PAGE profile, which featured 45 kDa subunits, and a specific activity of less than 10% of the activity of catalase T. This may have been attributable to a low haem/protein ratio in the enzyme isolated. Catalase T is encoded by the *CTT1* gene (Spevak et al., 1983), and the A protein by *CTA1* (Cohen et al., 1985). The primary structures are known from the corresponding base sequences (Hartig et al., 1986; Cohen et al., 1988). Both catalases occur aerobically, but not under fermentation conditions (Zimniak et al., 1976).

E. coli has two haemoproteins that have catalase properties, HPI (hydroperoxidase I) and HPII (hydroperoxidase II). HPII is an unconventional catalase with a subunit molecular mass of ~92 kDa (Loewen and Switala, 1985). Its prosthetic group is haem d_{cis}, a stereoisomer of the prosthetic group of the *E. coli* anaerobic terminal oxidase cytochrome *bd* (Chiu et al., 1989). HPII occurs in both the periplasmic space and the cytosol (Heimberger and Eisenstark, 1988). Its amino acid sequence closely resembles those of eukaryotic catalases (Loewen, 1992). HPII subunits are encoded at the *katE* locus of the *E. coli* chromosome, and the gene has been sequenced (Loewen et al., 1985; von Ossowski et al., 1991).

This paper compares the behaviour of four catalases (mammalian, yeast A and T, and microbial) towards NADPH as a cofactor and as a modulator. We employ the peroxide generation method that was pioneered by Chance (1950) in his study of peroxide compounds, but which was also used by Keilin and Hartree (1955) to classify peroxidatic activities and by Nicholls (1964) to analyse catalase compound II kinetics. It is shown that NADPH does not belong in any of the donor classes described by Keilin and Nicholls (1958), but has a unique redox behaviour. We put forward an hypothesis of NADPH action towards mammalian and yeast (but not *E. coli*) catalases which extends the previous model for the bovine enzyme (Hillar and Nicholls, 1992). Some of the results presented herein were the subject of a preliminary communication at the 1993 U.S. Biophysical Society meeting in Washington, D.C. (Hillar and Nicholls, 1993).

MATERIALS AND METHODS

Bovine liver catalase

Semicrystalline bovine liver catalase (BLC) (2 × crystallized, suspension in water containing 0.1%, v/v, thymol) (Sigma Chemical Co., St. Louis, MO, U.S.A.) was diluted in an appropriate buffer (50 mM potassium phosphate, pH 6.5 or 7.0) and centrifuged in a bench-top centrifuge for 5 min to remove insoluble material. For fluorescence studies, the protein was passed down a Sephadex G-25 column (1.5 × 10 cm) in 50 mM potassium phosphate buffer, pH 7.0. The catalase concentration was estimated by u.v. spectroscopy [ϵ_{406} 120 $mM^{-1} \cdot cm^{-1}$ for enzyme haematin (Nicholls and Schonbaum, 1963)].

Catalase from baker's yeast

The enzyme was isolated according to a modification of the procedure of Seah and Kaplan (1973). The following procedures were carried out at 4 °C. (1) Commercial active dry baker's yeast (60 g) (Fleischmann's) was homogenized (high-speed blender) for three periods of 15 s. The resulting powder was added to 200 ml of 50 mM Tris/HCl, 1 mM EDTA, 0.1% (w/v), phenylmethanesulphonyl fluoride, pH 8.0, and the mixture stirred for 30 min. Portions (50 ml) were transferred to 250 ml of poly(vinyl chloride) centrifuge bottles containing glass beads (0.4–0.5 mm diameter, 1 g/ml of solution) and agitated (side-arm shaker) for 2 h, centrifuged at 6000 *g* for 30 min and recentrifuged (15 min) to give a crude extract. (2) One volume of ethanol/chloroform (1:1, v/v) was added to 5 vol. crude extract in a 250 ml separatory funnel shaken for three 15 s periods. The contents were centrifuged at 12000 *g* for 30 min; the top aqueous layer was removed, and the middle (denatured protein) and bottom (organic solvent) layers discarded. (3) Solid $(NH_4)_2SO_4$ was added to 40% saturation and the sample centrifuged (12000 *g* for 30 min). The precipitate was discarded and the supernatant brought to 60% saturation. The precipitate was redissolved in 5 mM potassium phosphate buffer, pH 7.0, and $(NH_4)_2SO_4$ added to 45% saturation. The precipitate from a further centrifugation (12000 *g*, 30 min) was discarded, the supernatant brought to 60% saturation, and the resulting precipitate dissolved in 1–2 ml of 5 mM potassium phosphate buffer, pH 7.0. (4) The solution was passed down a Sephadex G-75 column (1.5 cm × 30 cm) pre-equilibrated with 5 mM potassium phosphate, pH 7.0 and eluted with 5 mM potassium phosphate, pH 7.0, utilizing a very slow flow (~ 1 ml/20 min). Catalase activity appeared with the initial protein peak. Fractions were pooled, the enzyme precipitated with $(NH_4)_2SO_4$ (80% saturation) and dissolved in 1.5–2.0 ml of 5 mM potassium phosphate buffer, pH 7.0. The brown solution was desalted on a 0.9 cm × 15 cm Sephadex G-25 (coarse) column, and eluted with 5 mM potassium phosphate buffer, pH 7.0. Aliquots (1.5 ml) with catalase activity were pooled. (5) Hydroxy apatite (HTP dry gel; Bio-Rad, Richmond, CA, USA) was rehydrated in 0.1 M potassium phosphate buffer, pH 7.0, packed in a 2.0 × 5.0 cm column and the pooled fractions applied. Protein was eluted stepwise with 20 ml of 0.1 M potassium phosphate buffer, pH 7.0, and 30 ml of each of 0.15, 0.2 and 0.3 M potassium phosphate buffer, pH 8.0. Catalase A eluted at 0.15 M, and catalase T at 0.20–0.30 M buffer. The fractions were each tested for activity, concentrated with Centriprep-30 centrifugal concentrators (Amicon, Danvers, MA, U.S.A.) to a total volume of ~ 0.5 ml, and stored at −20 °C. Yeast enzyme concentrations were obtained by u.v. spectroscopy [catalase A, $\epsilon_{408}^{1\,cm}$ (per haematin) 100 $mM^{-1} \cdot cm^{-1}$; catalase T, $\epsilon_{406}^{1\,cm}$ (per haematin) 100 $mM^{-1} \cdot cm^{-1}$ (Seah et al., 1974; Sharma et al., 1989)].

Escherichia coli HPII protein

Growth conditions, cell harvesting, and isolation and purification of HPII were as described by Loewen and Switala (1985). Lyophilized product was dissolved in 50 mM potassium phosphate buffer, pH 7.4 or 7.0. Insoluble material was removed by centrifugation, and the pH adjusted with 0.5 M monobasic/dibasic potassium phosphate buffer stocks. HPII catalase concentration was estimated by u.v. spectroscopy ($\epsilon_{405}^{1\,cm}$ per haematin 118 $mM^{-1} \cdot cm^{-1}$, Dawson et al., 1992) or by direct haematin determinations.

Pyridine haemochromogens

Reduced pyridine haemochromogens were prepared according to Fuhrhop and Smith (1975). Pyridine (100 μl) was added to 100 μl of sample in 50 mM potassium phosphate, pH 6.5, in a serological tube, and mixed for 1 min to induce denaturation. An aliquot (50 μl) of 1 M NaOH was then added and the sample diluted with water. Spectra were obtained in black-walled semi-micro cuvettes (1 cm, 0.5 ml) using a DU-50 spectrophotometer (Beckman Instruments Ltd.) with microbeam attachment, linked

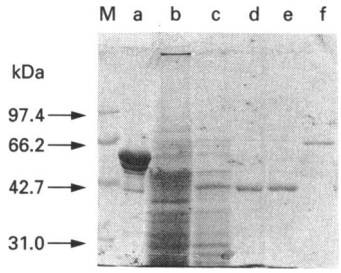


Figure 1 SDS/PAGE characterization of yeast catalases

The polyacrylamide electrophoretogram was stained with Coomassie Brilliant Blue R250 protein stain. Running conditions and gel composition were as described in the Methods and materials section. Lanes a–c each contain ~ 25 μg of protein, lanes d–f 4–5 μg of protein. Molecular-mass standard sizes are indicated in lane M. Lanes b–f were loaded with protein obtained during a typical yeast catalase purification. Lanes a, BLC; b, the crude extract from purification of the *S. cerevisiae* catalases; c, the ethanol/chloroform fraction; d, the G-75 Sephadex-isolated fraction; e, the hydroxyapatite-isolated catalase A fraction; and f, the hydroxyapatite-isolated catalase T fraction. Note that yeast T isoenzyme (lane f) runs as 66 kDa subunits, whereas yeast A isozyme (lane e) runs as 42 kDa subunits, presumably as a result of autolysis.

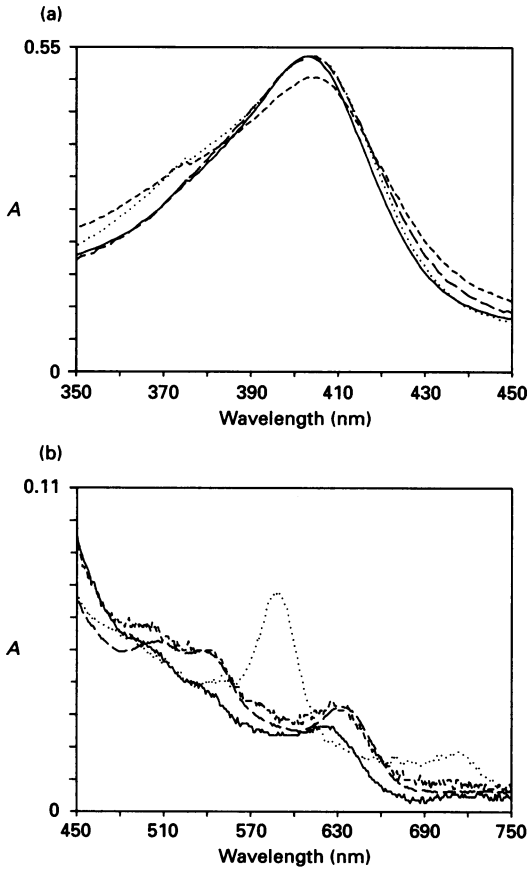


Figure 2 Absorption spectra of bovine liver, *S. cerevisiae*, and *E. coli* HPII catalases

The spectra are shown of BLC (4.3 μM haematin; continuous line), yeast A catalase (3.2 μM haematin, ——), yeast T catalase (16.1 μM haematin; – – – –), and HPII wild-type (4.5 μM haematin, dotted line) in 50 mM potassium phosphate, pH 7.0, at 25 °C for (**a**) the Soret region and (**b**) the visible region. Spectra are scaled for approximate absorbance equality at 750 nm. The original full-scale absorbances at the Soret maxima (**a**) were 0.52 for BLC, 0.33 for yeast A catalase, 1.65 for yeast B catalase, and 0.55 units for HPII respectively. The scale in **b** is expanded 5-fold.

to a 286-based computer with Beckman Dataleader software. The samples were reduced with a few crystals of sodium dithionite before being analysed.

Protein determination

Protein concentrations were determined by the method of Bradford (1976), using the Commassie Blue system according to the manufacturer's specifications (Bio-Rad Laboratories Ltd.). BSA was used as a protein standard. $A_{595}^{1\,cm}$ of samples and standards were measured in triplicate in a DU-7HS spectrophotometer (Beckman Instruments).

Enzyme activity and spectrophotometry

Assays for catalase activity were performed according to Beers and Sizer (1952). H_2O_2 concentration was determined by u.v. spectroscopy using $\epsilon_{240}^{1\,cm}$ 39.4 $M^{-1} \cdot cm^{-1}$ (Nelson and Kiesow, 1972). Catalase peroxide compound II formation was followed at 435 nm in pH 6.5 potassium phosphate, using 1 ml semi-micro black-sidewall quartz cuvettes.

SDS/PAGE

SDS/PAGE was performed according to Laemmli (1970). A Mini-Protean II slab gel (Bio-Rad Laboratories) was used, with 12% (w/v) acrylamide. Gels with ~ 25 μg of protein per sample well were run at 200 V in Tris/glycine, pH 8.3. Protein was visualized using Coomassie Blue. Figure 1 compares the mobilities of BLC (lane a) with baker's-yeast fractions obtained during purification (lanes b–f). The BLC band heterogeneity may be the result of incomplete denaturation. Yeast catalase T (lane e) shows 66 kDa subunits whereas catalase A (lane f) runs as 42 kDa subunits, presumably as a result of autolysis. This confirms the results of Seah et al. (1974), who obtained values of 60 kDa and 45 kDa for T and A catalases respectively.

Affinity chromatography

Affi-gel Blue (Bio-Rad Laboratories) was diluted in 10 mM Tris/HCl, pH 7.4, packed on a 1.1 cm × 1.5 cm column equilibrated with the same buffer, and washed with 2.5 ml of 1 mM NaN_3 and ~ 10 bed volumes of starting buffer. For elution, ~ 300 μg of protein (in 0.2–0.5 ml) was loaded and the column was washed with 10 ml of buffer, followed by 2.5 ml of buffer plus 1 mM ADP, another 10 ml of buffer, 1 ml of buffer containing 10 mM NADPH, another 10 ml of buffer, and a final aliquot containing NADPH. Fractions (0.5 ml) were monitored at 405 nm for catalase, at 280 nm for protein (no dinucleotide present) and at 340 nm after NADPH addition to monitor dinucleotide elution.

Fluorescence

Samples were diluted in 20 mM Tris/HCl, 0.1 mM EDTA, pH 8.0, according to Lowry et al. (1957). Emission spectra from 360–560 nm, excitation 340 nm, of 0.2 μM catalase samples at 30 °C were obtained in a 3 ml quartz cell. The Perkin–Elmer LS-50 luminescence spectrometer was linked to a 386SX computer with the Perkin–Elmer FLDM system.

Materials

Glucose oxidase (*Aspergillus niger*, Sigma Chemical Co., type X) was prepared in 50 mM potassium phosphate buffer and diluted to ~2 nM in the buffer used. D-Glucose (BDH, Poole, Dorset, U.K.) was used as a 0.2 M solution in distilled water. Potassium

ferrocyanide (BDH Chemicals) was used as a 10 mM solution in distilled water, and frozen until use.

RESULTS

Spectroscopy of beef liver, HPII, yeast T and yeast A catalases

Figure 2 compares the spectra of BLC with yeast catalases T and A, and with *E. coli* HPII. BLC has a Soret peak (Figure 2a) at 403–405 nm, and visible maxima (Figure 2b) at 622, 540 and 500 nm (see Nicholls and Schonbaum, 1963). Yeast catalases, which are protohaem-containing enzymes like BLC, have Soret maxima at 405 nm and visible peaks at 630, 535 and 505 nm (see Seah and Kaplan, 1973). Haemochromogen measurements did not support the idea that catalase A has very low absorption coefficients (results not shown). The two yeast enzymes are spectroscopically similar. HPII (see Claiborne et al., 1979) has a Soret band at 403 nm, and visible maxima at 588 and 712 nm.

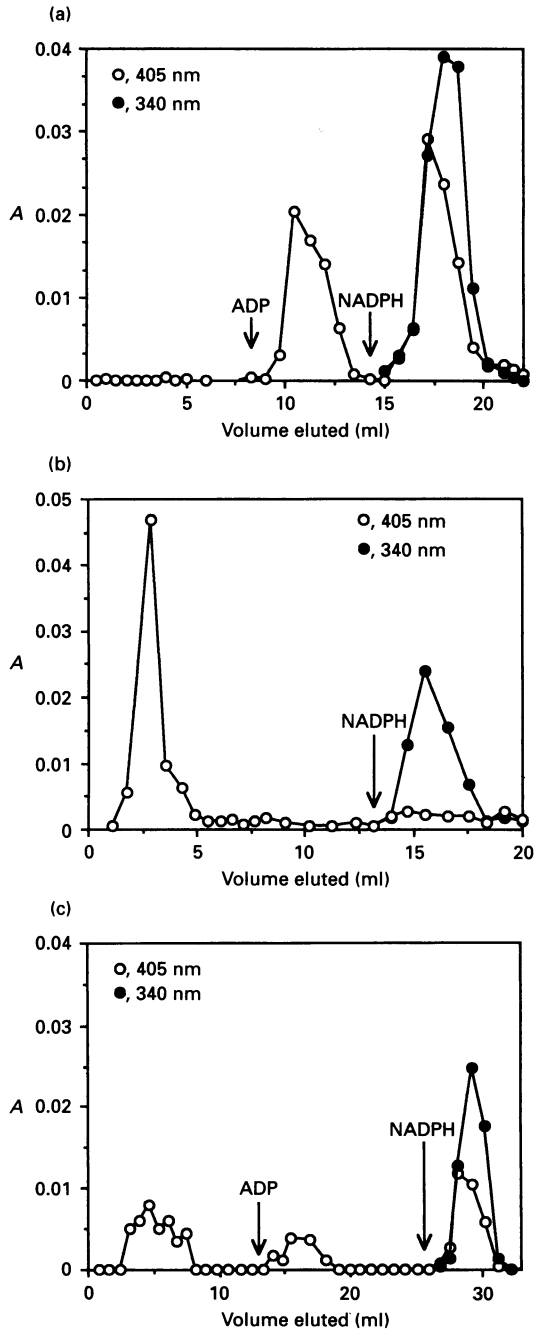


Figure 3 Elution profiles of bovine, yeast catalase and HPII wild type protein from an Affi-gel blue affinity column

Column preparation and elution conditions were as described in the Materials and methods section. The temperature was 4 °C. For each elution, 300 μg of catalase protein was applied. Haemoprotein (405 nm) and NADPH (340 nm) eluting absorbances were monitored. The samples applied were (**a**) a bovine enzyme sample, (**b**) an HPII wild-type enzyme sample, and (**c**) a yeast T catalase sample.

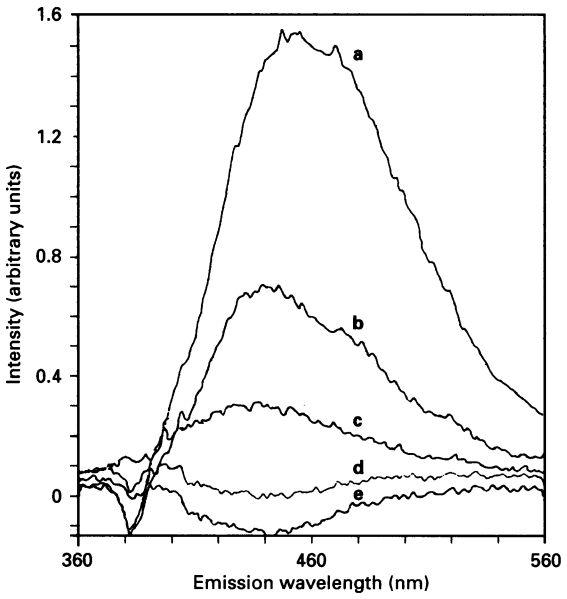


Figure 4 Difference fluorescence emission spectra of catalases

Spectra of bovine (**a**, **b**), HPII (**d**, **e**) and yeast catalase T (**c**) proteins are shown with the background buffer emission signal subtracted. The excitation wavelength was 340 nm. All samples contained 1 μM haematin in a final volume of 2.7 ml of 50 mM potassium phosphate, pH 7.0, at 30 °C. Other conditions were as described in the Materials and methods section. (**b**, **e**) Untreated proteins. (**a**, **c**, **d**) Proteins incubated for 30 min with 50 μM NADPH in sample buffer, then passed over a Sephadex G-25 column to remove excess NADPH.

Catalase binding of NAD(P): affinity chromatography

The enzymes were fractionated on a dye-linked affinity chromatography column (Thompson et al., 1975) specific for NAD(H)/NADP(H)-binding proteins, which was similar to one used previously for BLC (Jouve et al., 1986). Elution of BLC (Figure 3a) gives a dinucleotide-binding protein containing little or no native-bound NAD/NADP. Both ADP and NADPH induced significant release of this protein from the column, the major peak eluting with NADPH. Jouve et al. (1986) reported that a significant 'α' fraction, representing dinucleotide-loaded catalase, eluted with the starting buffer. Our fractions correspond only to those that were designated 'β', which elute with the dinucleotide or at very high salt levels. The elution profile of HPII catalase is shown in Figure 3(b). The pattern is typical of a non-dinucleotide-binding protein, in that all of the Soret absorbance elutes with the sample buffer. An NADPH peak then elutes without appreciable Soret absorbance. Figure 3(c) shows elution of yeast catalase T; a similar pattern was obtained for yeast catalase A (results not shown). In each case, both α and β fractions were eluted. The more substantial peaks were eluted

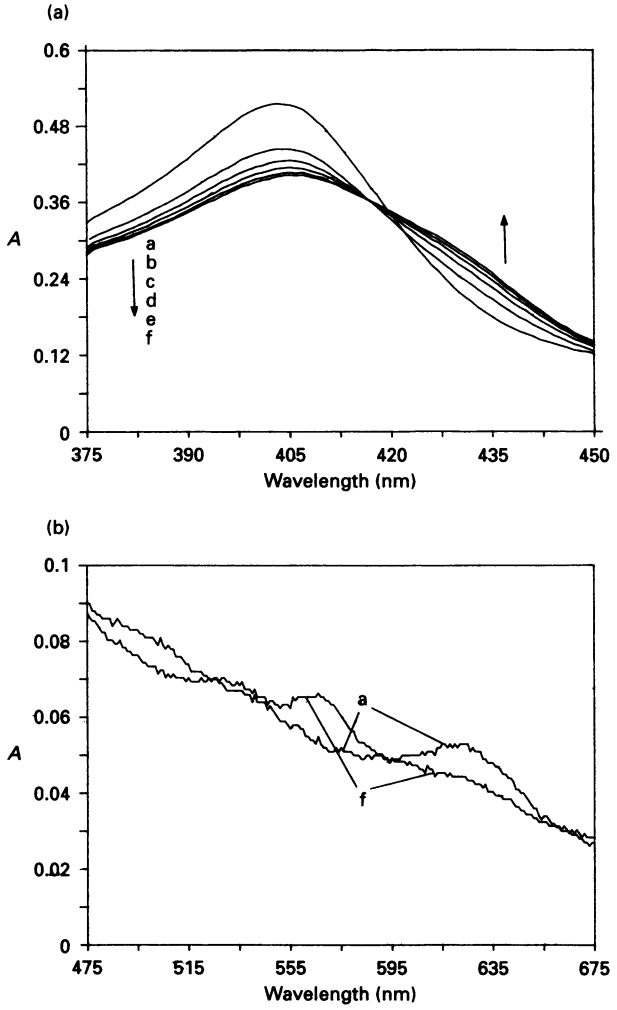


Figure 5 Formation of bovine catalase peroxide compounds

Absorbance spectra in the (**a**) Soret region and (**b**) visible region are shown for 4.3 μM haematin BLC in a final volume of 1 ml of aerobic 10 mM potassium phosphate, pH 6.5, with 2.0 nM glucose oxidase, at 25 °C. H_2O_2 generation was initiated by addition of 4 mM glucose. The arrows indicate the direction of spectral shifts from zero time to 60 min: **a**, 0 min; **b**, 12 min; **c**, 24 min; **d**, 36 min; **e**, 48 min; and **f**, 60 min.

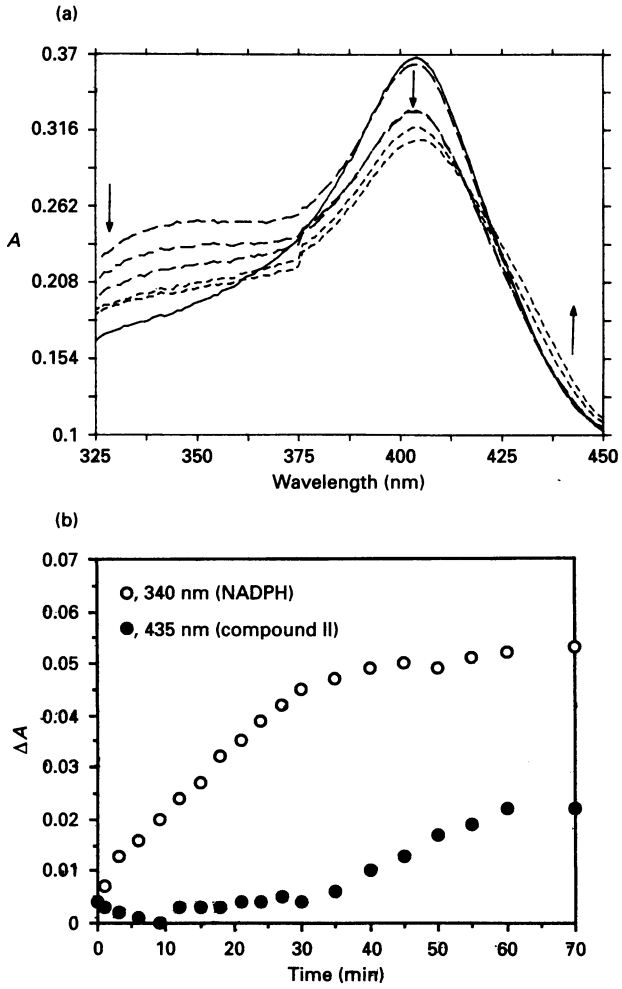


Figure 6 Oxidation of added NADPH by bovine liver catalase

(**a**) Spectral changes (Soret and near-u.v. regions). BLC (3.0 μM haematin) was dissolved in aerobic 10 mM potassium phosphate buffer, pH 6.5, plus 2 nM glucose oxidase at 25 °C (final volume 0.2 ml). The initial spectrum (continuous line) was taken before NADPH addition. The following spectra were taken after incubation with 10 μM NADPH plus glucose (2 mM). Arrows indicate the direction of the spectral shifts up to 70 min after addition of glucose. Spectra at 0, 12 and 24 min (——) indicate no compound II formation; spectra at 45 min and 70 min (– – – –) show formation of compound II. (**b**) Time course of the reaction. A secondary plot of the data from (**a**) shows the parallel changes at 340 nm (NADPH) and 435 nm (compound II). ΔA values are plotted versus time, with the direction of change at 340 nm inverted for comparison purposes.

with NADPH, as with BLC. Elution of β fractions with ADP and/or NADPH shows that both isoenzymes bind dinucleotides.

Catalase binding of nicotinamide adenine dinucleotides: fluorescence

Fluorescence emission spectra using 340 nm excitation showed a broad near-u.v. feature in commercial BLC, but not in HPII or yeast catalase T. NADPH is lost during purification and storage, although some commercial samples retain dinucleotide through tight binding (Kirkman and Gaetani, 1984). Our gel fractionation of BLC, however, showed no such α fraction; the near-u.v. signal in this enzyme does not derive from NADPH but from another fluorophore with related characteristics (see the Discussion section).

Binding of NADPH was examined by NADPH exposure of each enzyme for ~30 min. Free coenzyme was then removed by G-25 Sephadex filtration. The fluorescence spectra recorded before enzyme exposure to NADPH were subtracted from those obtained after exposure. The resulting difference spectra, which eliminate all the signals attributable to other fluorophores, are shown in Figure 4. Yeast and beef liver catalases take up the nucleotide, whereas HPII cannot do so.

Beef liver and yeast catalase peroxide compound II formation

Figure 5 shows the effect of continuously generated H_2O_2 upon BLC. Immediate compound I formation is seen at 403 nm; the subsequent red shift (Figure 5a) is caused by compound II production (see Kirkman et al., 1987; Hillar and Nicholls, 1992). In the visible region (Figure 5b), absorbance increases in the green region also signal the appearance of this species (Nicholls and Schonbaum, 1963).

Figure 6(a) shows a corresponding time course when catalase is preincubated with NADPH before H_2O_2 exposure. Spectral

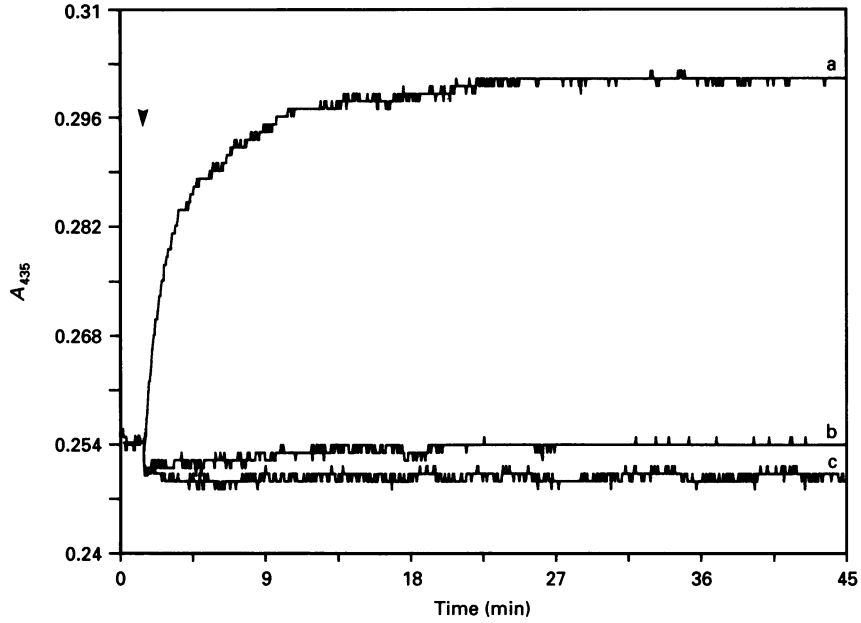


Figure 7 Effect of NADPH and ethanol upon formation of BLC compound II

BLC (4.0 μM haematin) was incubated in aerobic 10 mM potassium phosphate buffer, pH 6.5, with 2.0 nM glucose oxidase at 25 °C (final volume 0.8 ml). H_2O_2 generation was initiated by addition of 4 mM glucose (single arrowhead). Compound II formation is shown (**a**) in a control experiment, (**b**) in the presence of 20 μM NADPH, and (**c**) in the presence of 20 mM ethanol.

changes in the Soret (400 to 430 nm shift) and near-U.V. (340 nm decline) demonstrate concomitant NADPH oxidation as the catalase reaction proceeds. NADPH prevents compound II formation but is progressively oxidized by the enzyme. When all coenzyme has been oxidized, compound II formation begins. Figure 6(b) plots the absorbance changes at 340 nm and at 435 nm. The initial rate of NADPH oxidation is similar to the final rate of compound II formation.

Spontaneous formation of mammalian catalase compound II is caused by 'endogenous' hydrogen donors. Either ethanol or NADPH blocks the reaction, but in very different concentration regimes (Figure 7). At an NADPH concentration only slightly greater than that of the catalase (haematin), a lag phase in compound II formation is seen (Figure 7, trace b) during which NADPH oxidation occurs (Hillar and Nicholls, 1992). The duration of the lag depends upon the NADPH concentration, provided that the latter is greater than a minimal near-stoichiometric level (Hillar and Nicholls, 1992). Preaddition of NAD^+ or $NADP^+$, instead of NADPH, had no effect upon compound II formation (results not shown). NADH was partially active but NADPH was the more effective dinucleotide.

Ferrocyanide does not reduce compound II to ferricatalase but specifically accelerates compound II formation from compound I (Nicholls and Schonbaum, 1963). When BLC is preincubated with NADPH, the acceleration of compound II formation by ferrocyanide is eliminated (Hillar and Nicholls, 1992). NADPH is a more potent inhibitor of compound II formation than is ethanol; the two donor concentrations used in Figure 7 differ by three orders of magnitude. The rates of compound II decay, following addition of either ethanol or NADPH after ferrocyanide, are similar (results not shown). Neither ethanol nor NADPH reduces compound II directly (Kirkman et al. 1987; Hillar and Nicholls, 1992).

Yeast catalase T forms peroxide compounds like those of the bovine liver enzyme during continuous H_2O_2 generation. Ferrocyanide increases compound II formation without affecting compound I formation, whereas ethanol immediately reacts with the latter to give ferricatalase. Figure 8(a) (trace a) shows compound II formation by catalase T. The effect of preaddition of NADPH is shown in trace b. A typical NADPH oxidation-dependent lag phase is apparent. Figure 8(b) shows the effects of adding ferrocyanide and subsequently NADPH on catalase A. NADPH prevents *S. cerevisiae* catalase compound II formation and reduces its steady-state level, as it does with the bovine enzyme.

Similar experiments could not be carried out with *E. coli* HPII catalase because we have so far been unable to identify the appearance of a secondary peroxide compound in this enzyme, either under conditions of slow peroxide generation or by peracetate addition (P. Nicholls and A. Hillar, unpublished work). These observations are in agreement with those of Beaumont et al. (1990), who have proposed that NADPH binding and function may be intimately related to the propensity of the given catalase to form an oxoferryl compound II species.

DISCUSSION

NAD(P) binding and conservation of the catalase dinucleotide fold

This is the first report of NADPH binding to fungal catalases. Our findings with mammalian catalase were similar to those obtained previously (Jouve et al., 1986; Kirkman et al., 1987). In dye-affinity chromatography, NADPH was more effective than ADP in eluting the bound catalase. No α fraction was observed, and commercial bovine enzyme is probably NADPH depleted. It can, however, be reloaded with NADPH. *E. coli* HPII catalase showed no chromatographic evidence of a dinucleotide-binding site. Yeast catalases T and A had similar elution patterns and both bind NAD(P)H. NADPH is the preferred binding species in each case.

Fluorescence studies partially confirmed these conclusions.

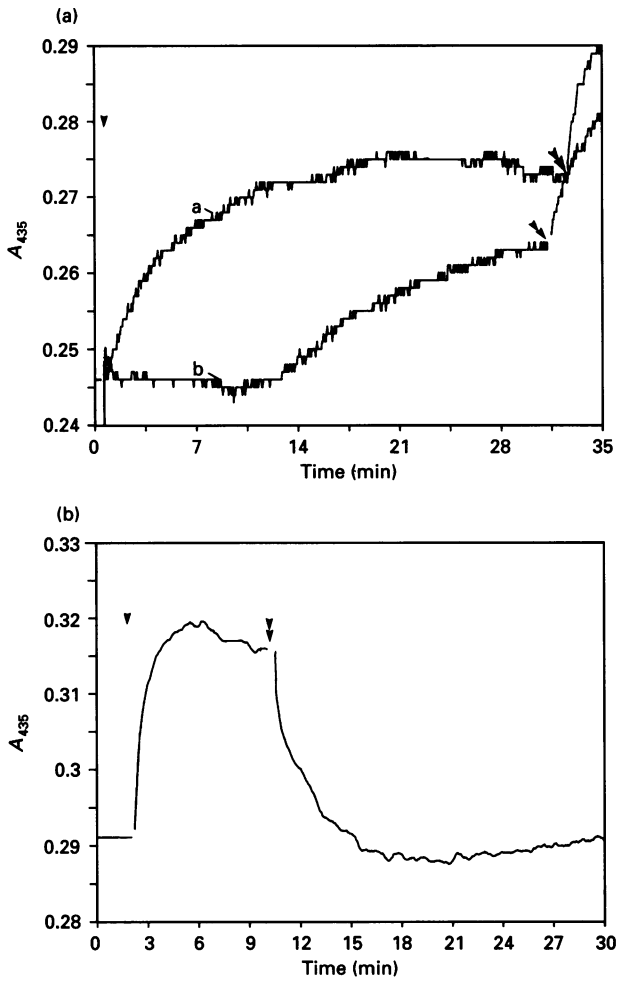


Figure 8 Effects of ferrocyanide and NADPH upon formation and decay of compound II of *S. cerevisiae* catalases

(**a**) Yeast catalase T (4 μM haematin) was incubated in aerobic 10 mM potassium phosphate buffer, pH 6.5, plus 5 nM glucose oxidase at 25 °C, in a final volume of 0.2 ml. Glucose (4 mM) was added (single arrowhead) to initiate peroxide compound formation. $K_4Fe(CN)_6$ (16 μM) was then added (double arrowhead) to increase the compound II level. Trace a shows the control rate of compound II appearance, trace b shows the effect of preaddition of 13 μM NADPH on compound II formation. (**b**) Yeast catalase A (2.3 μM haematin) was preincubated as in (**a**), together with 25 μM $K_4Fe(CN)_6$. Addition of 4 mM glucose (single arrowhead) initiated compound II formation. NADPH (20 μM) (double arrowhead) was added after the formation of compound II. Note that the trace was smoothed using a Savitzky–Golay function over 15 data points per calculation.

Bovine catalase showed an intrinsic emission at 440–460 nm, 20 nm below the expected fluorescence emission maximum for NADPH free in solution. Although its origin remains uncertain, the several types of bile pigment present in liver catalase (Nicholls and Schonbaum, 1963) suggest an artefact. In contrast, HPII catalase and yeast catalase T show no such fluorescence. Enzyme purification and storage probably result in autoxidation of any bound dinucleotide.

NADPH exposure followed by removal of excess nucleotide gave an authentic NADPH fluorescence with bovine enzyme. HPII after NADPH exposure gave no more fluorescence than untreated HPII. Yeast catalase T showed a significant fluorescence increase following NADPH treatment. Whereas *S. cerevisiae* catalases bind NAD(P)H as does mammalian enzyme, catalases of *A. niger* and *Penicillium vitale* may not (Melik-Adamyan et al., 1986). No general pattern has emerged among the fungal catalases. *A niger* catalase (Kikuchi-Torii et al., 1982) is unconventional, being a glycoprotein with a flavodoxin-like domain, and the catalase of *P. vitale* is also anomalous. Investigation of other fungal catalases will be needed to provide a more conclusive generalization.

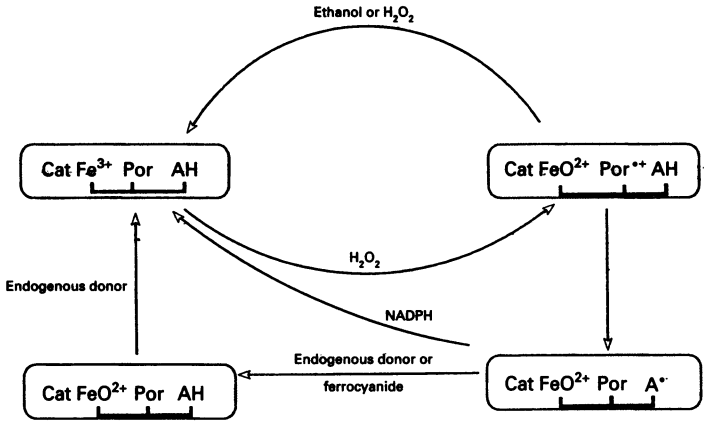
NADPH and NADH as unique two-electron donors to compound II: free-radical formation and migration

NADPH bound to catalase prevents compound II formation (Kirkman et al., 1987). It must act by somehow donating only one electron to regenerate a ferric state. NADPH oxidation to $NADP^+$ is however a two-electron process. This paradox needs resolution (see Almarsson et al., 1993). Of the six hydrogen donor groups identified by Keilin and Nicholls in 1958, three prevent compound II appearance, either by accelerating reduction of this species or by removing the precursor compound I in a direct two-electron step. The latter reagents, like alcohols, typically work in the millimolar range, because they must compete with peroxide for compound I. In contrast, NADPH and NADH act at micromolar levels to prevent compound II formation, but do not react rapidly with compound I. They therefore do not resemble alcohol donors. Phenols accelerate both formation and decomposition of compound II, and NADPH is oxidized by plant peroxidases by a similar mechanism (De Sandro et al., 1991); however, the failure of NADPH to affect catalase compound II decay shows it does not act like the phenol group. Sodium nitrite reduces compound II in one-electron steps and also prevents compound II formation by reducing compound I directly to ferric enzyme (Nicholls, 1964). NADPH/NADH does not show such behaviour.

It is concluded that NADPH and NADH comprise a unique hydrogen-donor class because (i) they do not react with compound I directly, (ii) they block compound II formation by ferrocyanide and by endogenous donors, and (iii) they are oxidized more slowly than other two-electron donors but at a rate identical with that of compound II formation in their absence. Scheme 1 presents a model incorporating these features. A protein radical species is initially formed by spontaneous decay of compound I porphyrin radical. This oxidizing radical can either oxidize another component of the system or can be reduced by one of the electrons from NADPH, while the other reduces the ferryl iron to its ferric state. This process could function by a concerted mechanism or via an additional radical intermediate. Almarsson et al. (1993) have recently proposed an elaborate model which involves one-electron oxidations and which extends the previous hypothesis of Kirkman et al. (1987). In our view, however, a concerted two-electron mechanism would be more parsimonious and thus more acceptable.

NADPH thus reacts not with compound I *per se* but with its immediate one-equivalent decay product (the hypothetical protein radical). It also blocks ferrocyanide from donating an electron to the haem. Although ferrocyanide and NADPH compete for the compound I decay product, NADPH binding is sufficiently tight ($K_d \sim 10$ nM) to eliminate the one-electron step. NADPH is oxidized slowly because the rate is dependent upon the concentration of the $CatFeO^{2+}PorA^{\cdot}$ species (Scheme 1). It is therefore similar to the rate of compound II formation.

Dolphin et al., (1971) proposed that both catalase and peroxidase compound I have a ferryl haem iron with the second oxidizing equivalent present as a porphyrin radical. If the porphyrin radical accepts electrons from local amino acid residues, radical migration will be initiated. Protein radicals are



Scheme 1 Catalase peroxide compound formation and decay in the presence and absence of NADPH: the action of endogenous donors and ferrocyanide

$CatFeO^{2+}Por^{\bullet+}$ indicates compound I, in which the two equivalents are shared between the iron atom and the porphyrin ring (see text); $catFeO^{2+}$ indicates compound II. AH represents the postulated oxidizable amino acid residue close to the haem group, which becomes $A^{\bullet}$ in the postulated intermediate that reacts with NADPH.

implicated in the mechanisms of other haemoproteins, notably myoglobin and cytochrome *c* peroxidase (Wilks and Ortiz de Montellano, 1992; Ortiz de Montellano, 1992; Miller et al., 1992; Arduini et al., 1992).

Although metmyoglobin does not normally engage in catalysis, H_2O_2 oxidizes its haem iron to a ferryl [Fe^{IV}=O] form. The second oxidation equivalent produces a transient protein radical in horse and sperm-whale metmyoglobin (Catalano et al., 1989; Davies, 1991), horse and human haemoglobin, and leghaemoglobin (Davies and Puppo, 1992). Site-directed mutagenesis of yeast cytochrome *c* peroxidase implicates Trp-191 in the generation of a protein radical (Miller et al., 1992). Trp-191 replacement by phenylalanine causes one radical e.p.r. component to disappear (Fishel et al., 1991), though not the entire signal. Other centres, perhaps tyrosine residues, may therefore exist in cytochrome *c* peroxidase and free-radical migrations could occur between these sites and the haem group.

The formation of radicals in myoglobins and cytochrome *c* peroxidase provides support for a similar process in catalases. Bound NADPH may donate electrons in a concerted reaction before an initial radical can migrate and oxidize a remote component. The distance from the catalase haem edge to bound NADPH is ~ 1.4 nm (14 Å) (Fita and Rossmann, 1985a,b). Radical migration over a distance of 1.4 nm (14 Å) is not prohibited; such a process can occur between Tyr-151 and haem in myoglobin. One catalase tyrosine (Tyr-214) is available in the NADPH-binding cavity and several more are available in the β-barrel 'walls' of the haem pocket.

How radical migration in catalase may occur remains speculative. Support for the general hypothesis has recently come from resonance Raman spectra of peroxidase and catalase compounds I (Chaung and Van Wart, 1992). Resonances of tyrosines are enhanced by ferric–tyrosine charge transfer (Chaung et al., 1988). These modes are enhanced by compound I excitation, when the π-cation radical may be delocalized onto the proximal Tyr-357 ligand.

Site-directed mutagenesis could indicate whether or not tyrosine residues are implicated directly in formation and migration of radicals in catalases. As *S. cerevisiae* catalases bind NADPH, this can be investigated in a system characterized genetically (Hartig et al., 1986; Cohen et al., 1988). Another approach is to take a prokaryotic catalase that does not bind NADPH, such as *E. coli* HPII, and carry out site-directed mutagenesis to create an NADPH-binding site *de novo*. Both types of experiment are being planned.

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