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Reaction of *E. coli* catalase HPII with cyanide as ligand and as inhibitor

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Abstract

Cyanide forms an inhibitory complex with the haem d-containing *E. coli* catalase HPII, spectrally similar to the cyanide complex of beef liver enzyme but with absorption bands shifted 90 nm towards the red end of the spectrum. Both the K_d and K_i values are ≈ 7 μM in the wild-type enzyme. The cyanide reaction is slow, with a bimolecular 'on' constant approx. 2000 × smaller than that of eukaryotic enzyme, and an 'off' constant diminished by a similar amount. Catalases with a mutated distal histidine (H128) fail to bind cyanide at cyanide concentrations below 50 mM. Catalases with a mutated distal asparagine (N201) show only small changes in cyanide affinity from the wild type. The major fraction of HPII N201A has a $K_d \approx 40$ μM, and a minor fraction has a lower cyanide affinity; the major fraction of HPII N201Q has a $K_d \approx 15$ μM. The K_d and K_i for HPII N201D is ≈ 8 μM, essentially identical with that of the wild type but N201D appears to bind cyanide somewhat more rapidly than does wild-type enzyme. The HPII mutant N201H can be obtained in both haem d and protohaem forms; it exhibits two types of cyanide binding behaviour. In its protohaem form it binds cyanide poorly ($K_d \geq 0.25$ mM). After peroxide treatment converts it into haem d or a closely related species it binds cyanide with a much higher affinity ($K_d \approx 15$ μM). Cyanide binding to HPII requires a distal histidine to provide hydrogen-bonding stability, but not a distal asparagine. Rates of cyanide binding and release are controlled by haem group accessibility through the channel leading to the outside. In HPII N201H channel opening may depend upon oxidation of the haem from the starting protohaem to the final haem d form.

Keywords: Hydroperoxidase II; Heme d; Cyanide; Catalase; Ligand binding kinetics; (*E. coli*); (Bovine liver)

1. Introduction

Cyanide is a universal ligand for and inhibitor of haem enzymes. Its poisonous character is due to its action on cytochrome oxidase, and it also binds catalases, peroxidases, ferric forms of haemoglobin and myoglobin, and (slowly) cytochrome *c*. The rates and equilibria involved are very variable. Eukaryotic catalase and peroxidase bind cyanide rapidly, taking up both the anion and a proton as they do so [1]. Ferric ('met') haemoglobin and myoglobin bind more slowly and normally bind the anion [2]. Cytochrome oxidase binds the neutral form, like catalase and peroxidase, but reacts very slowly in the resting state [3]. Only in mixed valence and related activated

Abbreviations: BLC, beef liver catalase; HPII, hydroperoxidase II (catalase) of *E. coli*; EDTA, ethylene diamine tetraacetic acid.

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states does it bind cyanide rapidly, a phenomenon which has stimulated studies directed both at understanding cyanide inhibition and at elucidating the mechanism of the enzyme with cyanide as a 'probe' [4,5]. Van Buuren et al. summarised the classical similarities and differences in cyanide binding among several haem proteins (cf. Table 4 in Ref. [3]). Site-directed mutagenesis now allows directed changes in the amino-acid components of the haem pockets of these enzymes to determine the role of side chains in ligand binding [6,7].

The homotetrameric catalase from *E. coli*, HPII, is evolutionarily homologous with the similar homotetrameric eukaryotic enzyme as well as with enzymes from other prokaryotes that contain protohaem as their prosthetic group. But HPII has a 'd' haem derived from a doubly *cis*-hydroxylated protohaem [8]. The third pyrrole is modified and the hydroxyl group adjacent to a propionate side chain is linked to the latter via a lactone bridge [9]. This extra ring structure is approximately at right angles to the haem plane. The channel to the haem iron is more restricted than that in beef liver catalase and the enzymatic activity somewhat lower [10–12].

The present paper describes the cyanide binding behaviour of HPII catalase and some site-directed mutants. Cyanide binding is much slower than with the protohaem enzyme although the affinity is similar and the anion is bound together with a proton as with protohaem catalases. Mutation of the distal histidine markedly inhibits cyanide binding by the enzyme but mutation of the distal asparagine has little effect. We conclude that, unlike binding of peroxide, cyanide binding does not involve hydrogen bonding with the distal asparagine but only with the distal histidine. The lower rates of binding and dissociation of cyanide may reflect greater steric hindrance in the channel leading to the haem. Similar final binding constants show that the haem d_{cis} structure does not affect the ability of the iron to bind low spin ligands.

2. Methods and materials

2.1. Materials

Beef liver catalase was from Boehringer Mannheim Biochemica Canada. The crystalline suspension was diluted in sodium borate/HCl buffer (pH 8.25), stirred for a few minutes at room temperature (25°C) to dissolve the crystals and the suspension then centrifuged with a bench centrifuge for 3 minutes to remove insoluble material. The resulting solution was diluted with potassium phosphate buffer (50 mM pH 7.4) to a concentration appropriate to each test conducted. The concentrations of catalase hematin were estimated using an extinction coefficient of 120 $mM^{-1}cm^{-1}$ at 406 nm [13].

Escherichia coli HP II catalases were prepared in the Department of Microbiology, University of Manitoba, according to Loewen and Switala [14]. Site-directed mutants were prepared as described by Loewen et al. [15]. Concentrations of HP II hematin were estimated using the extinction coefficient reported by Dawson and coworkers [16] of 118 $mM^{-1}cm^{-1}$ at 405 nm. The lyophilized protein was dissolved in potassium phosphate buffer and centrifuged to remove insoluble material.

Stock solutions of 0.2 M potassium cyanide (Mallinckrodt and BDH) were prepared and frozen until required. Fisher supplied the 30% hydrogen peroxide. KH_2PO_4 was a product of Baker and K_2HPO_4 a product of Caledon.

2.2. Catalase assay

The dismutation of hydrogen peroxide was followed spectrophotometrically [17] using an extinction coefficient for hydrogen peroxide at 240 nm of 39.4 $M^{-1}cm^{-1}$ [18]. Catalatic activity of HPII wild-type catalase was proportional to enzyme (heme) concentration with an overall rate constant of 1.3×10^6 $M^{-1}s^{-1}$ (Obinger et al., unpublished). HPII wild-type activity was also assayed in the presence of cyanide. As HP II binds cyanide much more slowly than BLC (see below), the enzyme was preincubated with cyanide in the assay medium for 5 min prior to peroxide addition. Measurements were carried out with either a Beckman™ DU-7HS or an Aminco™ DW-2 spectrophotometer, the former linked to an Apple™ GS computer and the latter to a Compaq™ 286 device with Olis™ software and hardware. Data were analysed using the Olis™ fitting routines for various types of exponential reaction or by transfer to Macintosh and fitting routines in the Deltagraph™ software graphics package.

2.3. Catalase spectroscopy

The spectra of HPII (wild-type) enzyme and its cyanide complex are compared in Fig. 1a. The absorbance maxima of free enzyme in both the Soret and visible regions agree with reported literature values ± 2 nm [14]. The rate of cyanide binding by HPII was slow enough to be monitored using an Aminco™ DW-2 spectrophotometer with a data acquisition rate of 1 point/second, followed by the analysis described. Similar rates were obtained from time courses of the spectrophotometric assay if

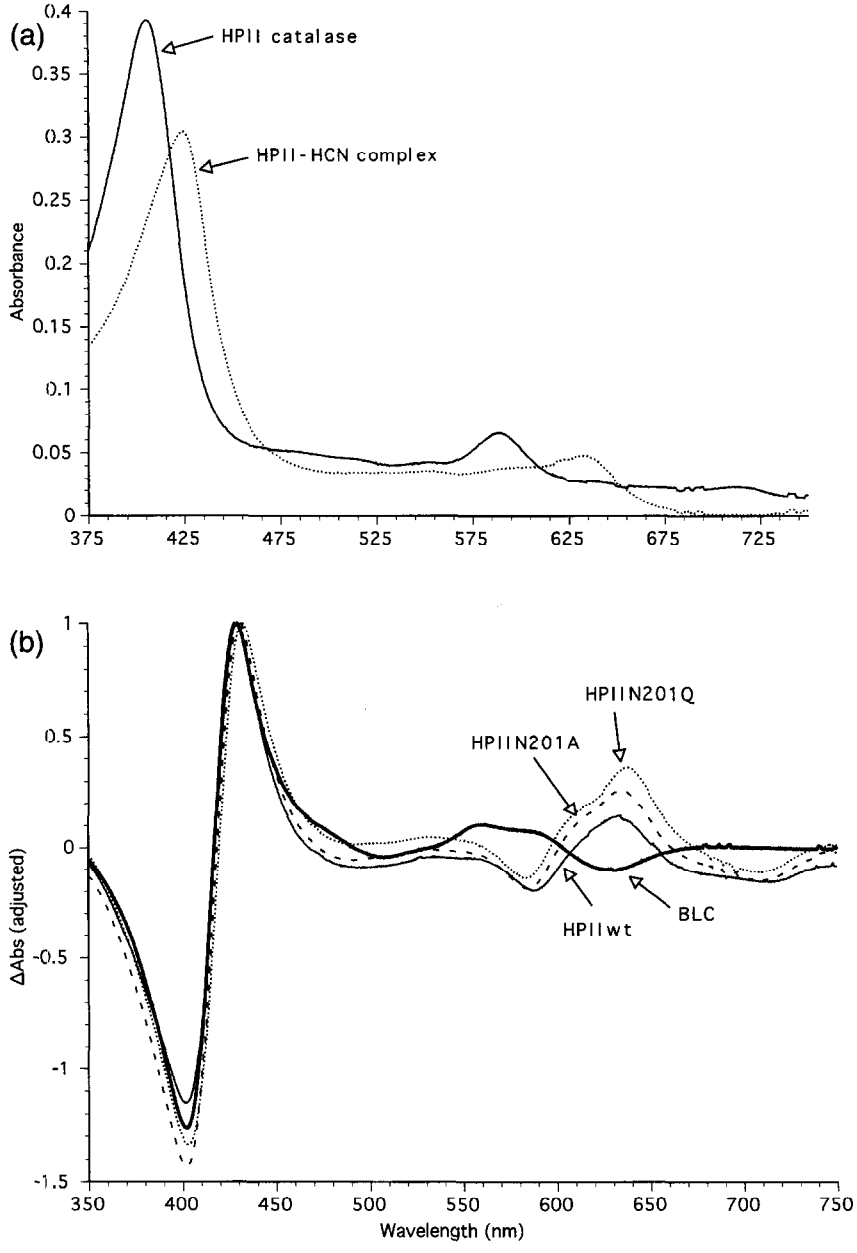

Fig. 1. Spectral changes in wild-type and mutant HPII catalases upon the binding of cyanide. (a) Absolute spectra of wild-type enzyme and its cyanide complex. 3.3 μM HPII catalase (haem concentration) in 50 mM potassium phosphate buffer (pH 7.4) at 30°C, in the presence and absence of an excess of cyanide added as KCN. (b) Difference spectra of four catalase cyanide complexes. Conditions as in Fig. 1a. Absorbance differences adjusted to 1.0 at the Soret maximum in each difference spectrum. Beef liver enzyme (BLC), HPII wild type (HPIIwt) and the mutants N201Q and N201A are shown.

cyanide was added to the reaction mixture after commencement of the assay. Unless otherwise indicated all experiments were performed at 30°C in 50 mM potassium phosphate buffer at pH 7.4.

3. Results

Fig. 1a shows the spectral changes in wild-type HPII which occur upon addition of cyanide. The peaks at 590 and 715 nm (weak) are replaced by a single peak at 630 nm. This resembles the high to low spin transition which occurs when beef liver or other eukaryotic catalases [13] are treated with cyanide (Fig. 1b). The charge transfer bands at 500 and 622 nm are replaced by a single band at 555 nm. We therefore presume that the corresponding changes in HPII also reflect the replacement of charge transfer (high-spin) bands by a low-spin band. The bands are displaced about 90 nm towards the red end of the spectrum — a consequence of the haem ring being of a 'haem d' type (a diol or spirolactone derivative state). Very similar spectral changes are seen when the two mutant enzymes N201Q and N201A are treated with cyanide (Fig. 1b). The characteristic spectral changes are independent of the presence of the essential asparagine in the haem pocket.

The binding is monophasic, with an 'n' value of 1.0, and a K_d of about 7 μM at pH 7.4 (Fig. 2a). As with the mammalian enzyme [13] the K_d is almost independent of pH in the neutral range, indicating that the bound species is HCN rather than CN^-. Cyanide is also an effective inhibitor of the catalytic reaction. The 'Dixon plot' of the reciprocal of the rate against the inhibitor concentration in Fig. 2b shows that this inhibition is also described by a simple binding algorithm. The resulting K_i is almost identical to the K_d value obtained spectrophotometrically with reagent levels of enzyme present (Fig. 2a).

HPII thus resembles eukaryotic enzyme in its affinity for cyanide and its binding of the protonated form in a reaction that does not involve any interactions between the four haem groups in the tetrameric enzyme. The reaction differs from that with beef liver and other eukaryotic-type enzymes in being slow. As shown in Fig. 3a, the binding can be tracked kinetically with a standard spectrophotometer. each addition of cyanide requires a finite time for equilibration

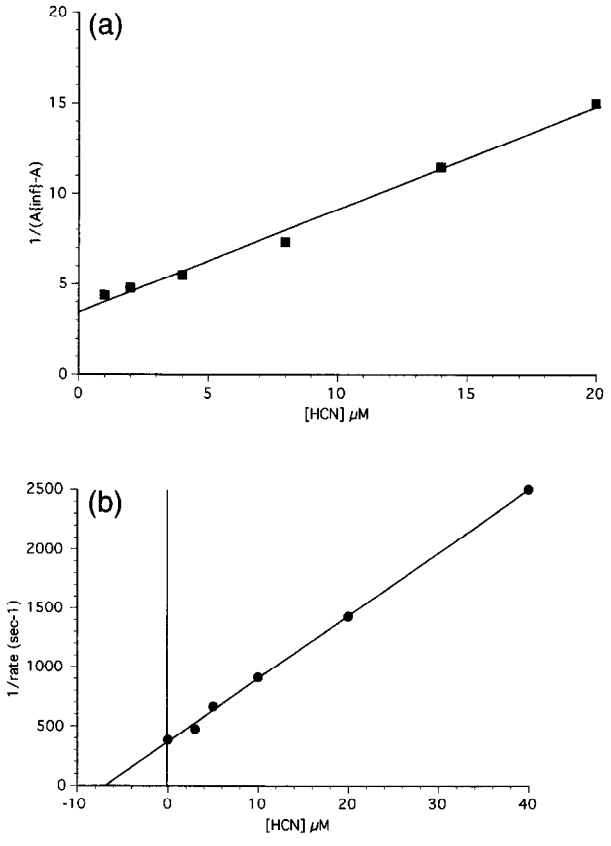

Fig. 2. Equilibrium binding of wild-type HPII and inhibition by cyanide. (a) Spectroscopic titration with cyanide. Plot of the reciprocal of the absorbance difference against the cyanide concentration. Note that: $1/(A_{inf} - A) = [HCN](1/K_d A) + (1/A)$ for simple cyanide binding. Conditions are as in Fig. 1. (b) Inhibition of enzyme activity by cyanide. Assays were carried out at pH 7.0 as described in Section 2, in the presence of varying amounts of cyanide. The rate constants for peroxide decomposition are plotted against [HCN].

with the enzyme. Fig. 3b shows the data in the form of a Guggenheim plot (apparent rate constants vs. HCN concentration). The process is a simple exponential reaction with a rate constant between 500 and 800 $M^{-1}s^{-1}$ depending upon the sample of HPII used. The catalytic inhibition induced by cyanide was also slow. Reactions using the polarographic (O_2 release) or spectrophotometric (H_2O_2 decay) assays showed that cyanide inhibition takes time, with a rate dependent on cyanide concentration in the same way as for ligation (results not shown).

Table 1 compares the cyanide binding behaviour of two types of mutant — those with a mutated distal histidine residue (H128) and those with a mutated

distal asparagine (N201). The histidine 128 mutants are protohaem proteins. Their enzyme activity is essentially zero [15], and thus they do not undergo the catalytic cycles needed for haem conversion [8]. They also bind cyanide very poorly, with K_d values in the 10–100 mM range. The N201 mutants, however, which are haem d proteins capable of turnover, show high cyanide affinity (Fig. 4). Their cyanide binding is in the micromolar range (Table 1). Fig. 4a shows a Hill plot of cyanide binding to the N201A mutant. This has a apparent K_d of 40 μM, but part of the population shows an even lower cyanide affinity ($\approx$ 0.3 mM). Fig. 4b shows the corresponding plot for N201Q; this enzyme form shows a cyanide

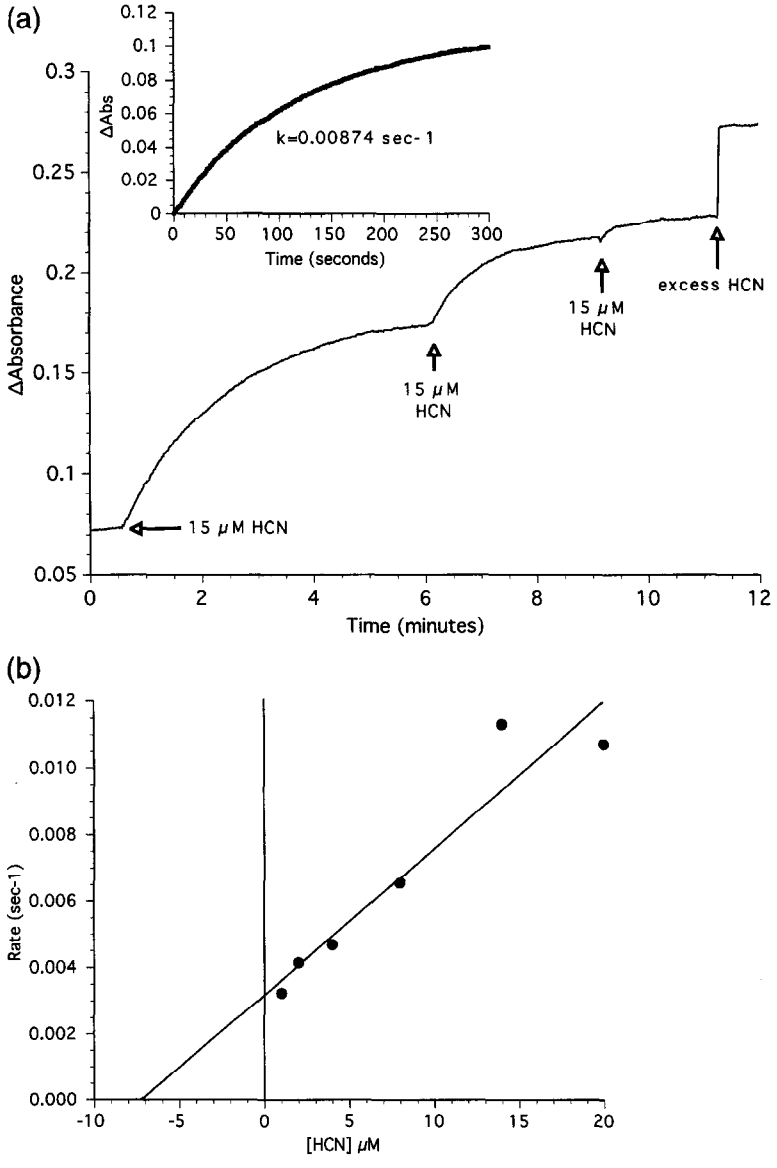

Fig. 3. Kinetics of cyanide binding to HPII. (a) Time-course of cyanide binding during a titration of enzyme with inhibitor. Additions of 15 μM aliquots of KCN are shown. Conditions as in Fig. 2a. Inset shows an exponential fit of the initial data. (b) Guggenheim plot: cyanide reaction rate (s^{-1}) as a function of HCN concentration. Data from initial cyanide additions in experiments as in Fig. 3a are used, the reciprocal of the fitted rate constant in each case being plotted against [HCN]. The slope of the line is that for the rate constant of between 500 and 800 $M^{-1}s^{-1}$, the exact value of which depends upon the actual sample of HPII used. The intercept of 0.003 s^{-1} represents the rate of cyanide dissociation from the haem group.

affinity of 15 μM with a small fraction of lower ($\approx$ 0.3 mM) affinity.

The mutant N201D has a cyanide binding K_d of 7.5 μM and a K_i between 6 and 9 μM, essentially the same as that of wild-type enzyme (Fig. 5a). Substitution of aspartate for asparagine in the haem pocket has no effect upon cyanide affinity although catalytic activity is markedly diminished [10,15]. The rates of cyanide binding to N201 mutants are also slow like the wild-type enzyme. But binding to N201D was slightly faster than to the wild-type enzyme (and the rate of dissociation was correspondingly also increased). Fig. 5b illustrates the cyanide binding to N201D. Some of the resulting rate constants are listed in Table 1.

The mutant N201H is especially interesting [15]. It exists in both protohaem and haem d forms. The former is the form isolated from the cells; the latter is the form produced by incubation with peroxide-generating systems. Cyanide binding to both species can therefore be examined. Fig. 6a shows the spectra obtained with the two forms as prepared and with excess cyanide. Fig. 6b plots the results of two titrations with cyanide, the first with the protohaem species, and the second with the haem d form. The protohaem form binds cyanide poorly and often biphasically, with a higher and a lower affinity phase. The average binding affinity as illustrated in Fig. 6b is over 0.25 mM. The binding rate is, however, approximately the same as that of the wild type (not shown). The major effect contributing to the low affinity is thus probably a larger 'off' constant. The final spectrum in Fig. 6a is not identical with that of isolated wild-type enzyme and may represent a precursor of the final haem d species rather than the spirolactone haem d proper. When converted into this form of the enzyme, however, the N201H mutant binds cyanide with a $K'_d \approx 16$ μM, as shown in Fig. 6b. The affinity has increased and is no longer significantly less than that of the wild type, and the reaction rate remains slow, like that of the wild type.

4. Discussion

The K_d for cyanide binding to wild-type *E. coli* catalase measured spectroscopically is nearly identical with the K_i measured catalytically. This implies the presence of an appreciable amount of free enzyme during catalytic turnover, as with mammalian enzyme [19]. Cyanide is a probe for the reaction of the enzyme with its substrate both in this way and in Chance's method in which cyanide is added to the steady state enzyme-peroxide compound mixture [20].

Cyanide binding to wild-type and mutant enzymes is slow, whether measured directly or by following inhibition of the catalytic reaction. In this respect HPII is like myoglobin and haemoglobin and unlike eukaryotic catalase or peroxidase. The channel leading to the haem may be deeper and relatively occluded in the bacterial enzyme [10]. On the other hand, the absence of any large effect of pH on cyanide binding rates and equilibria over the pH range from 5.0 to 7.4 indicates that it is the undisso-

Table 1
Cyanide binding by *E. coli* and beef liver catalases

Catalase	K_d (binding)	K_i (inhibition)	k_{on}
Beef liver [a]	8 μM	10 μM	9×10^5 M^{-1} s^{-1}
HPII wild type	7 μM	7 μM	500–800 M^{-1} s^{-1}
H128N	> 10 mM	ND	ND
N201A	40 μM	$\approx$ 20 μM	ND
N201D	8 μM	8 μM	$\approx$ 1700 M^{-1} s^{-1}
N201Q	15 μM	ND	ND
N201H protohaem	$\geq$ 300 μM	ND	$\approx$ 50 M^{-1} s^{-1}
N201H haem d_{cis}	15 μM	ND	433 M^{-1} s^{-1}

The behaviour of HPII wild type, a distal his mutant(H128N) and several Asn (N201) mutants is compared with that of classical eukaryotic enzyme. pH 6.8–7.4, 25–30°C. K_d, K_i and k_{on} are reported with respect to total HCN (the bound species includes both anion and proton). Experiments with different enzyme samples typically give values $\pm$35% of those cited.

[a] Chance [1,20]; Wolfe et al. [19].

ciated form of cyanide (hydrocyanic acid) that binds initially, like eukaryotic catalases and peroxidases and unlike myoglobin and haemoglobin [1–3,20].

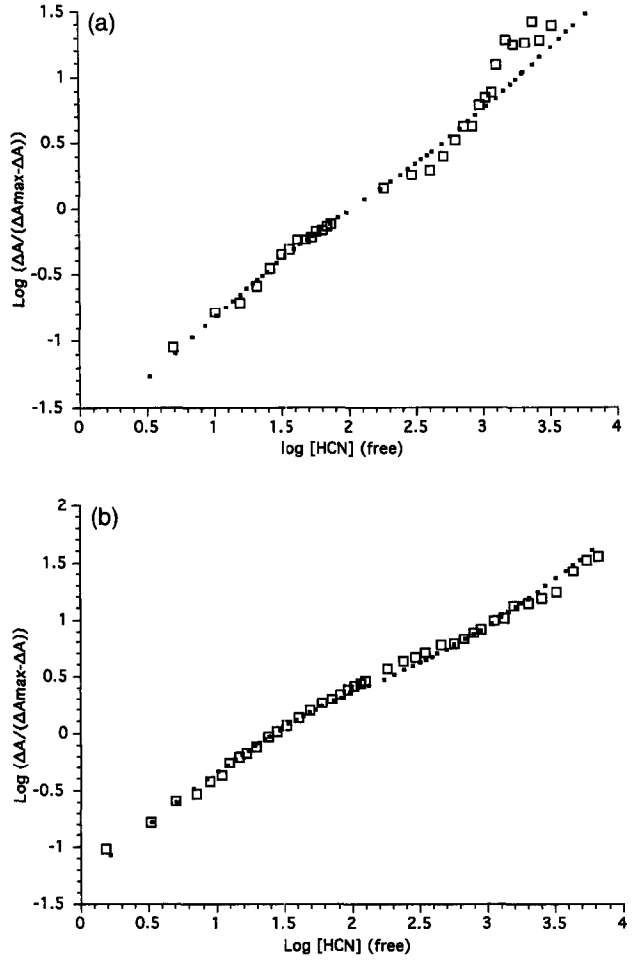

Fig. 4. Cyanide binding by two types of Asn-201 mutant. (a) Hill plot: cyanide binding by N201A. Open squares: data. Small closed squares: computer simulation of biphasic binding with 52% of the enzyme binding with a high affinity K_d of 35 μM (region A), and 48% with a low affinity K_d of 0.4 mM (region B). Free cyanide concentration was calculated as: Total[HCN] − (($\Delta A / A_{max}$)[E]) where [E] = total enzyme (haematin) concentration. Titration of 6.1 μM (haematin) enzyme was with aliquots of between 5 μM and 5 mM KCN at pH 7.4, 30°C. Other conditions are as in Fig. 1b. (b) Hill plot: cyanide binding by N201Q. Open squares: data. Small closed squares: computer simulation of biphasic binding with 75% of the enzyme binding with a high affinity K_d of 15 μM (region A), and 25% with a low-affinity K_d of 0.6 mM (region B). Free cyanide concentration was calculated as: Total[HCN] − (($\Delta A / A_{max}$)[E]) where [E] = total enzyme (haematin) concentration. Titration of 7.7 μM (haematin) enzyme was with aliquots of between 2.2 μM and 5 mM KCN at pH 7.4, 30°C. Other conditions are as in Fig. 1b.

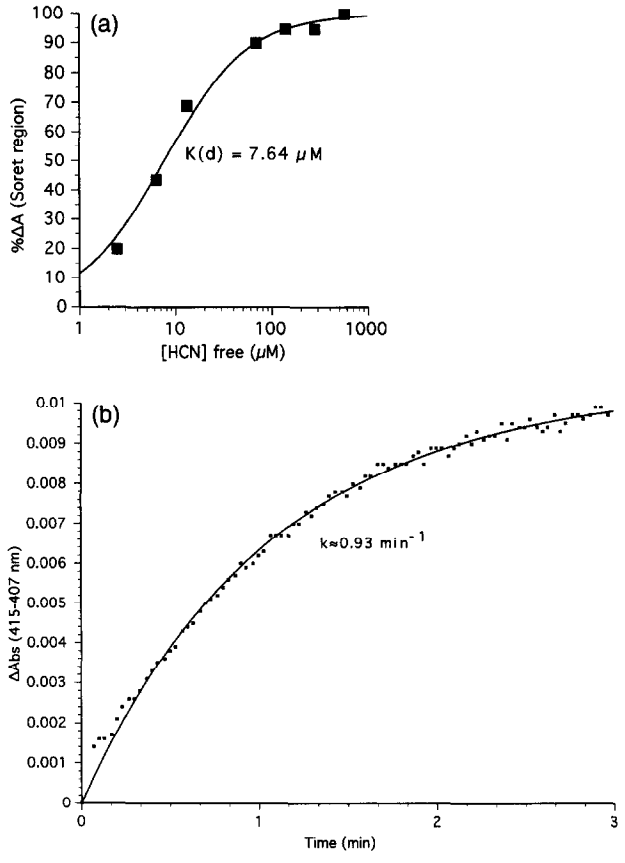

Fig. 5. Cyanide binding to N201D. (a) Equilibrium between N201D and cyanide. N201D has a K_d of 7.5 μM and a K_i of between 6 and 9 μM. Conditions are as in Fig. 1b. (b) Cyanide binding kinetics of N201D. N.B. the reaction is somewhat faster than that observed with wild type enzyme. The initial rate constant for the reaction with 1.3 μM HCN (the sum of k_{on}[HCN] + k_{off}) is 0.015 s^{-1} and the rate of cyanide dissociation from the haem group is thus $\approx$ 0.013 s^{-1} (cf. Fig. 3b).

The proton and the cyanide anion may, of course, adopt different ultimate locations in the enzyme molecule, and the possibility of H-bonding between the anion on the iron and the distal histidine remains an hypothesis.

The absence of effective binding to the H128N and H128A mutants shows that the hydrogen-bonding between ligand and distal histidine is important for stabilizing the cyanide complex. The ease of cyanide binding to the N201Q, N201A and N201D mutants suggests that although this residue may be important for peroxide activation [10,22] it does not modify cyanide binding in any significant manner — either rates or equilibria. N201H is an anomalous case and

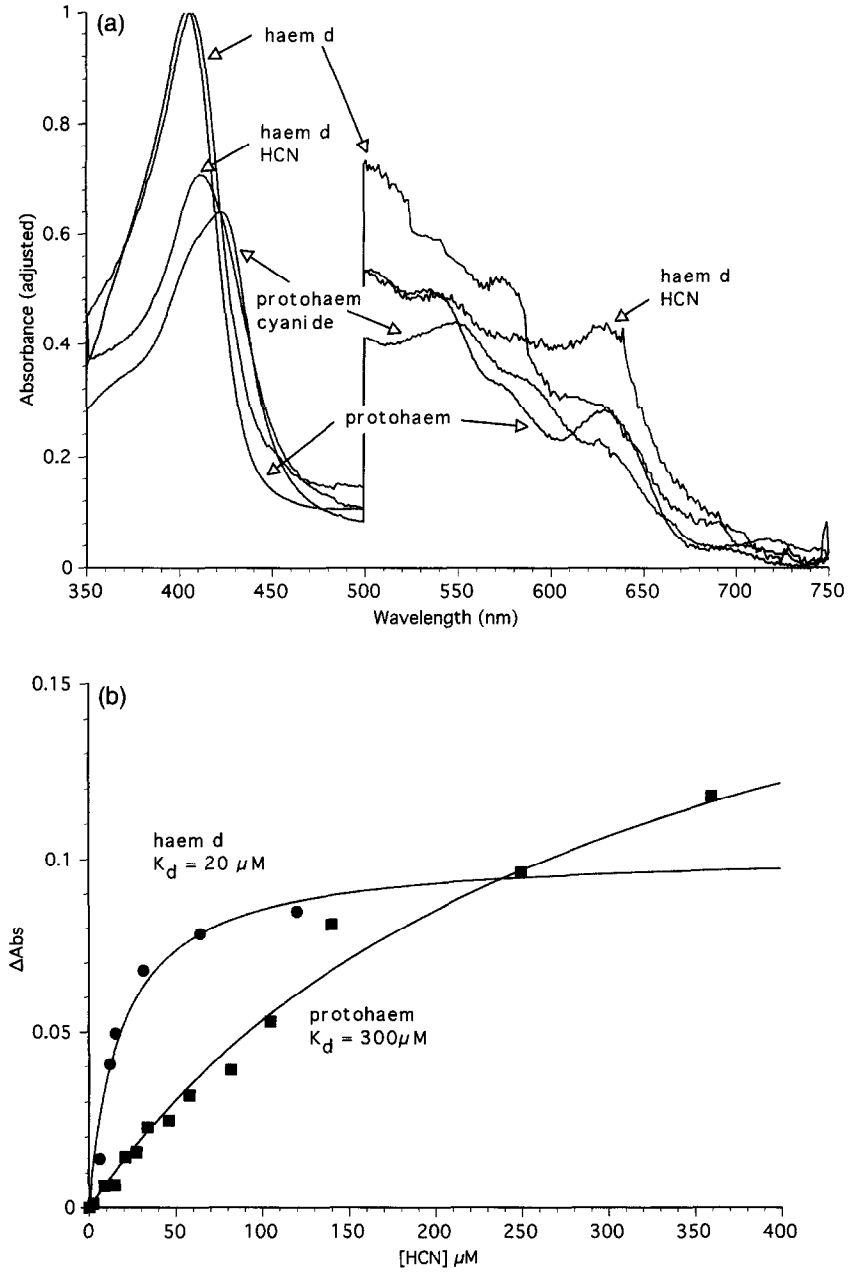

Fig. 6. Cyanide complexes of the protohaem and haem d forms of the HPII catalase mutant N201H. (a) Spectra of the two forms of N201H (prepared as described in Section 2 and the text) in the presence and absence of excess cyanide. (b) Binding equilibria for the two forms of N201H. Plots of the absorbance change (ΔAbs) against cyanide concentration are fitted to the equation: $\Delta\text{Abs} = \Delta\text{Abs}_{max} K_d/(K_d + [\text{HCN}])$. Conditions as in Fig. 1b and Section 2.

it permits an examination of the effect of haem type. In its protohaem form very poor cyanide binding is seen. As H128 is presumably available for H-bonding in this case, some conformational difficulty may be involved and relieved when the haem d species or one of its precursors is formed. An X-ray crystal structure of the N201H mutant may soon be available to check the conformational differences between this mutant and the wild-type protein [9].

We conclude that there are distinctly different effects of the distal residues H128 and N201 on cyanide binding with HPII. Neither K_d (the spectro-

scopic dissociation constant) nor K_i (the inhibition constant determined catalytically) are affected by mutating N201 but both cyanide binding and activity require an intact H128. This may be a difference from high-spin ligands like formate and fluoride the binding of which, like the catalytic activity, appears to be more sensitive to replacement of N201 [22,23]. Our conclusions concerning the effect of haem chemistry on low-spin ligand binding as exemplified by cyanide are that the change from protohaem to haem d_{cis} has very little effect. The haem pocket components, especially the distal histidine, and the groups lining the channel that leads to the haem are much more important [21]. The relationships between these effects and those upon catalytic activity will be examined in a subsequent paper [12].

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