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Kinetic and equilibrium studies of acrylonitrile binding to cytochrome *c* peroxidase and oxidation of acrylonitrile by cytochrome *c* peroxidase compound I

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ABSTRACT

Ferric heme proteins bind weakly basic ligands and the binding affinity is often pH dependent due to protonation of the ligand as well as the protein. In an effort to find a small, neutral ligand without significant acid/base properties to probe ligand binding reactions in ferric heme proteins we were led to consider the organonitriles. Although organonitriles are known to bind to transition metals, we have been unable to find any prior studies of nitrile binding to heme proteins. In this communication we report on the equilibrium and kinetic properties of acrylonitrile binding to cytochrome *c* peroxidase (CcP) as well as the oxidation of acrylonitrile by CcP compound I. Acrylonitrile binding to CcP is independent of pH between pH 4 and 8. The association and dissociation rate constants are $0.32 \pm 0.16\ M^{-1}\ s^{-1}$ and $0.34 \pm 0.15\ s^{-1}$, respectively, and the independently measured equilibrium dissociation constant for the complex is 1.1 ± 0.2 M. We have demonstrated for the first time that acrylonitrile can bind to a ferric heme protein. The binding mechanism appears to be a simple, one-step association of the ligand with the heme iron. We have also demonstrated that CcP can catalyze the oxidation of acrylonitrile, most likely to 2-cyanoethylene oxide in a "peroxygenase"-type reaction, with rates that are similar to rat liver microsomal cytochrome P450-catalyzed oxidation of acrylonitrile in the monooxygenase reaction. CcP compound I oxidizes acrylonitrile with a maximum turnover number of $0.61\ min^{-1}$ at pH 6.0.

1. Introduction

Ferric heme proteins tend to bind weakly basic ligands such as azide, cyanide, fluoride and imidazole [1–6]. Ligand binding is often pH dependent due to protonation/deprotonation of the ligands as well as the involvement of ionizable groups within the protein. In our continuing efforts to understand the reactivity differences between the globins and the peroxidases, we have compared the binding of azide, fluoride, and cyanide to metmyoglobin (metMb) and cytochrome *c* peroxidase (CcP) [7–12]. For both proteins, the protonated neutral forms of the ligands, HN_3, HF, and HCN, preferentially diffuse into the heme pocket, with the distal histidine facilitating deprotonation of the ligand and anion binding to the iron. The major difference in formation of the ligand/protein complexes between the two proteins is the fate of the ligand proton. In metMb, the proton is released to solution at neutral pH while the proton is retained in the CcP/ligand complex leading to significantly different pH dependencies of the dissociation rate constant and consequently, the equilibrium dissociation constant.

We have begun ligand binding studies with weak nitrogen bases such as imidazole and 1-methylimidazole, which form cations upon protonation, and with ligands such as organonitriles for which no proton association or dissociation phenomena would be expected in the pH range accessible to metMb or CcP. For the nitriles, any pH dependence of the ligand association and dissociation reactions can be attributed solely to properties of the protein. While there are many studies of imidazole binding to heme proteins [9,11,13–23], we have been unable to find any prior reports of nitrile binding to heme proteins, although binding of organonitriles to transition metals is well known [24,25].

We have found that CcP binds imidazole and 1-methylimidazole one to two orders of magnitude weaker than metMb and that the pH dependence of 1-methylimidazole binding to CcP is unusual, increasing with decreasing pH. The imidazole/CcP studies will

Abbreviations: ACN, acrylonitrile; CEO, 2-cyanoethylene oxide; CcP, generic abbreviation for cytochrome *c* peroxidase whatever the source; yCcP, authentic yeast cytochrome *c* peroxidase isolated from *S. cervisiae*; rCcP, recombinant cytochrome *c* peroxidase expressed in *E. coli*, which has an identical amino acid sequence to that of yCcP; CcP-I, cytochrome *c* peroxidase compound I; metMb, metmyoglobin.

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be published elsewhere (manuscript in preparation). In this paper we report on equilibrium and kinetic studies of acrylonitrile binding to CcP. We believe that these are the first equilibrium and kinetic studies of acrylonitrile binding to a heme protein.

Although we have not found any studies of acrylonitrile binding to heme proteins in the literature, there are several reports on the oxidation of acrylonitrile by cytochrome P450 indicating that acrylonitrile can bind within the heme pocket of cytochrome P450 [26–31]. Michaelis constants for acrylonitrile in the cytochrome P450-catalyzed monooxygenase reaction vary widely, from 52 μM [28] to 0.19 M [26]. In the second part of this communication, we show that CcP compound I can oxidize acrylonitrile with rates similar to those of rat liver microsomal cytochrome P450.

2. Materials and methods

2.1. Materials

Both authentic yeast cytochrome *c* peroxidase, yCcP, and a recombinant CcP with the exact amino acid sequence of yCcP, rCcP, were used in this study. Isolation and purification of CcP has been described previously [32,33]. Potassium phosphate salts, potassium acetate, and H_2O_2 (30%) were obtained from Fisher Scientific. H_2O_2 solutions were standardized by titration with Ce(IV) as described previously [34]. Acrylonitrile was obtained from Aldrich Chemical Company.

2.2. Buffers

Between pH 4.0 and 5.5, buffers were 0.010 M acetate with sufficient KH_2PO_4 to adjust the ionic strength to 0.100 M. Between pH 5.5 and 8.0, the buffers were mixtures of KH_2PO_4 and K_2HPO_4 with a total ionic strength of 0.100 M.

2.3. Spectroscopic measurements and protein concentration determination

Spectra of protein solutions were determined using a Varian/Cary Model 3E or a Hewlett Packard Model 8452A spectrophotometer. Protein concentrations were determined from the absorption spectra using the Soret extinction coefficient at pH 6: 98 mM^{-1} cm^{-1} at 408 nm for yCcP [32] and 101 mM^{-1} cm^{-1} at 408 nm for rCcP [35].

2.4. Equilibrium binding of acrylonitrile to CcP

Spectroscopic changes associated with the binding of the acrylonitrile to CcP enabled monitoring of complex formation. Determination of the equilibrium constants was done by titrating ~10 μM protein with increasing concentrations of buffered acrylonitrile solution. Equilibrium studies were carried out at 0.5 pH intervals between 4 and 8, using 0.100 M ionic strength buffers, 25 °C.

2.5. Kinetic studies of acrylonitrile binding to CcP

The rate of acrylonitrile binding to yCcP was determined using an Applied Photophysics Ltd. Model DX.17MV stopped-flow spectrophotometer. Reactions were carried out under pseudo-first order conditions with acrylonitrile in excess. Protein concentrations ranged between 1 and 5 μM. Rate constants were determined at a minimum of five different acrylonitrile concentrations at each value of pH. Kinetic studies were carried out at 0.5 pH intervals between pH 4 and 8, using 0.100 M ionic strength buffers, 25 °C.

2.6. Single-turnover kinetics of CcP compound I oxidation of acrylonitrile

CcP compound I was generated by the addition of a stoichiometric amount of H_2O_2 to CcP at pH 6.0, 0.100 M ionic strength. Aliquots of acrylonitrile were added to the compound I solution and the reaction monitored by observing the decrease in absorbance at 424 nm. Kinetic studies were carried out at pH 6, 0.100 M ionic strength, potassium phosphate buffer, 25 °C.

3. Results

3.1. Equilibrium binding of acrylonitrile by CcP

Addition of acrylonitrile to CcP causes significant changes in the spectrum of CcP consistent with binding of acrylonitrile to the heme iron, Fig. 1. The difference spectrum between CcP and CcP in the presence of 1.35 M acrylonitrile is shown in the lower panel of Fig. 1. Acrylonitrile binding can be monitored by observing the change in absorbance as a function of acrylonitrile concentration. A plot of the absorbance as a function of the acrylonitrile concentration is shown in the Supplementary material accompanying this article. The absorbance change can be described by a hyperbolic function of the acrylonitrile concentration, Eq. (1), where A_{obs} is the observed absorbance, A_0 is

$$A_{obs} = A_0 + \Delta A_{max} \frac{[ACN]}{K_D + [ACN]} \quad (1)$$

the absorbance in the absence of acrylonitrile, ΔA_{max} is the maximum absorbance change in the presence of infinite ligand, K_D is

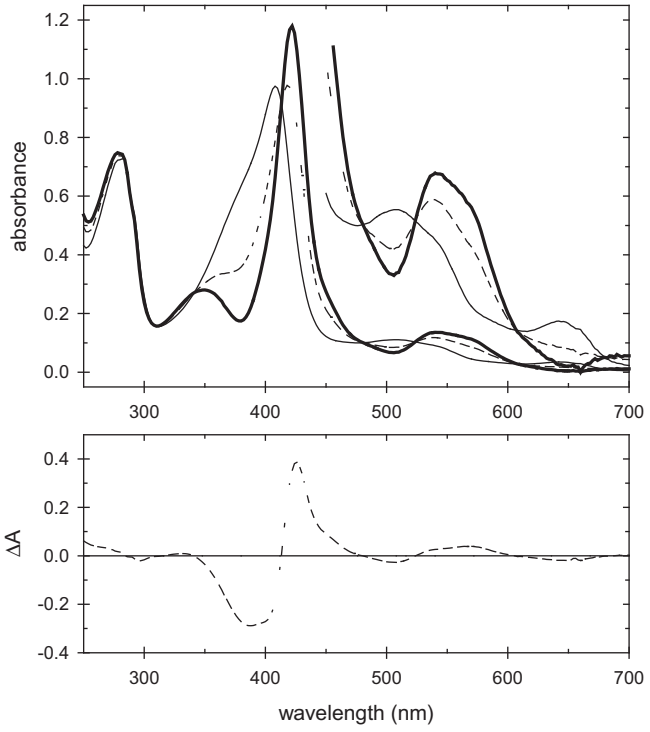

Fig. 1. The absorption spectrum of rCcP in the presence and absence of acrylonitrile. Top Panel. CcP (thin solid line), CcP in the presence of 1.35 M acrylonitrile (dashed line), Calculated spectrum of the acrylonitrile/CcP complex. (thick solid line). Bottom Panel. Difference spectrum between CcP and CcP in the presence of 1.35 M acrylonitrile. The maximum and minimum in the difference spectrum occurs at 426 and 386 nm, respectively. Experimental conditions: [rCcP] = 9.65 μM, pH 7.0, 0.100 M ionic strength, potassium phosphate buffer, 25 °C.

the equilibrium dissociation constant, and [ACN] is the free acrylonitrile concentration.

The value of K_D for the acrylonitrile/CcP complex was determined as a function of pH over the pH stability range of CcP, pH 4 to 8. The logarithm of K_D is plotted as a function of pH in the upper panel of Fig. 2. The value of K_D is independent of pH, averaging 1.1 ± 0.2 M for 28 independent determinations.

3.2. Spectrum of the acrylonitrile/CcP complex

The maximum solubility for acrylonitrile in aqueous buffer (~1.4 M) limits the amount of complex formed in solution to ~60% of the total enzyme. However, the spectrum of the complex can be calculated from the concentration dependence of the absorbance changes Fig. 1. The spectrum of the acrylonitrile/CcP complex is typical for a six-coordinate, low-spin heme protein, with α, β, Soret (γ), and δ bands at ~572, 540, 422, and 350 nm, respectively. The corresponding extinction coefficients are 11.3, 14.0, 122, and 29.0 mM^{-1} cm^{-1}, respectively.

3.3. Kinetics of acrylonitrile binding to CcP

The kinetics of acrylonitrile binding to CcP was investigated using stopped-flow techniques. Upon mixing excess acrylonitrile with CcP, a single exponential change in absorbance is observed as a function of time. The binding reactions are typically complete within about 1 min of mixing. Between pH 4.0 and 7.5, k_{obs} is a linear function of the acrylonitrile concentration, consistent with reversible binding of acrylonitrile to CcP, Eq. (2). For the mechanism shown in Eq. (2), the observed rate constant is given by Eq. (3).

$$E + ACN \underset{k_d}{\overset{k_a}{\leftrightarrows}} E \cdot ACN \tag{2}$$

$$K_{obs} = k_{a[ACN]} + k_d \tag{3}$$

The association (k_a) and dissociation (k_d) rate constants can be found from the slope and intercept of plots of k_{obs} as a function of acrylonitrile concentration (see Supplementary material). The logarithm of k_a and k_d are plotted as a function of pH in the lower panel of Fig. 2. Both k_a and k_d are independent of pH with average values of 0.32 ± 0.16 M^{-1} s^{-1} and 0.34 ± 0.15 s^{-1}, respectively.

The rate constants can be used to calculate K_D according to the binding mechanism shown in Eq. (2). Values of $K_D = k_d/k_a$ are plotted in the upper panel of Fig. 2 along with K_D determined from the equilibrium measurements. Averaging the kinetically determined value of K_D over the pH range 4.0 to 7.5 gives a value of 1.3 ± 0.9 M, within experimental error of the value for K_D of 1.1 ± 0.2 M determined from equilibrium measurements. The agreement between the kinetically-determined K_D value and K_D determined from the equilibrium studies is consistent with the simple binding mechanism shown in Eq. (2). Values of k_a, k_d and K_D as functions of pH are tabulated in the Supplementary material.

At pH 8.0, the binding of acrylonitrile to CcP is considerably slower than at lower pH. In addition, k_{obs} appears to be an autocatalytic function of the acrylonitrile concentration (Supplementary material). This change in binding is most likely related to the incipient alkaline denaturation of CcP and changes in the nature of the solvent at the high concentrations of acrylonitrile.

3.4. Oxidation of acrylonitrile by CcP compound I

Liver microsomes have been reported to catalyze the epoxidation of acrylonitrile as a major pathway for elimination of acrylonitrile in the body [28]. Reported maximum turnover rates range between 0.26 and 4.9 min^{-1} based on the total cytochrome P450 content of the microsomal preparations [26–31]. Since we have shown that acrylonitrile binds to the heme group in CcP, we decided to see if CcP could oxidize acrylonitrile. We have investigated the transient-state kinetics of acrylonitrile oxidation by CcP compound I. In these studies, CcP and acrylonitrile are mixed, forming an equilibrium mixture of the acrylonitrile/CcP complex. The reaction is initiated by the stoichiometric addition of H_2O_2. The reduction of CcP compound I by acrylonitrile is slow and the endogenous reduction of the oxidized sites in compound I compete with the reduction by acrylonitrile [36]. Fig. 3 shows plots of the absorbance at 424 nm as a function of time in the presence of various concentrations of acrylonitrile. At low concentrations of acrylonitrile, the reduction of compound I is very similar to that of the endogenous reduction. In the presence of higher concentrations of

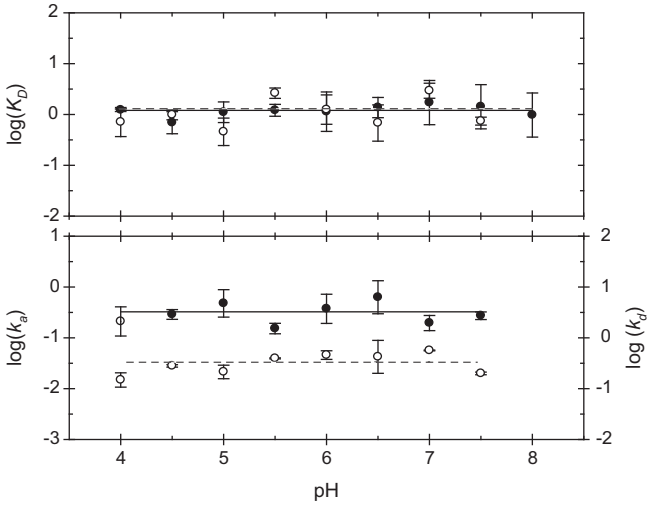

Fig. 2. Equilibrium and rate constants for acrylonitrile binding to CcP as a function of pH. Top Panel – Equilibrium dissociation constant, K_D, – solid circles. Kinetically determined $K_D = k_d/k_a$ – open circles. Bottom Panel – Association rate constant, k_a, – solid circles, left-hand axis. Dissociation rate constant, k_d, – open circles, right-hand axis.

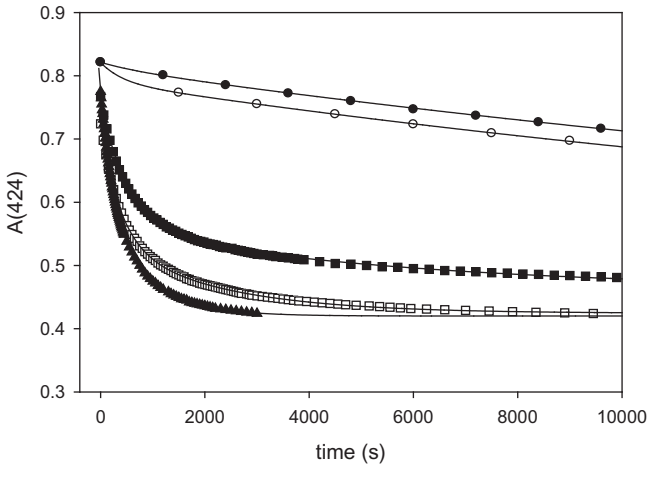

Fig. 3. Effect of acrylonitrile on the reduction of CcP compound I. The change in absorbance at 424 nm as a function of time is plotted at various concentrations of acrylonitrile, [ACN]. [ACN] = 0, solid circles; [ACN] = 0.061 M, open circles; [ACN] = 0.122 M, solid squares; [ACN] = 0.243 M, open squares; [ACN] = 0.608 M, solid triangles. Experimental conditions: Stoichiometrically-formed yCcP compound I (~10 μM), 0.100 M ionic strength buffers at pH 6.0, 25 °C.

acrylonitrile, reduction of compound I is significantly faster than the endogenous reduction, indicating that acrylonitrile reduces compound I and is, in turn, oxidized by compound I.

The endogenous reduction of CcP compound I is a biphasic kinetic process [36]. The rate constant for the fast phase of the reaction is $1.3 \times 10^{-3}\ s^{-1}$ and is attributed to the migration of the Trp-191 radical away from the heme pocket. The slow phase of the reaction has a rate constant of $2.9 \times 10^{-5}\ s^{-1}$ at pH 6 and is attributed to reduction of the ferryl site. If acrylonitrile is oxidized to the epoxide by CcP compound I, epoxidation would involve the transfer of the ferryl oxygen from compound I to acrylonitrile and is a two-electron oxidation. Direct oxidation of acrylonitrile by CcP compound I requires the participation of both oxidizing equivalents in the enzyme intermediate and we anticipated that the fast phase of the reaction will be most affected by acrylonitrile. In Fig. 4, the rate constant for the fast phase of the reduction of compound I, k_{R1}, is plotted as a function of the acrylonitrile concentration. The observed rates of reduction of compound I is significantly slower than the equilibration rate of acrylonitrile binding to CcP. We proposed a mechanism involving rapid equilibration of acrylonitrile (ACN) in the heme pocket of CcP compound I, followed by the rate-limiting oxygen transfer from the ferryl heme to the substrate, k_{cat}, and rapid dissociation of the product, 2-cyanoethylene oxide (CEO), from the active site, Eq. (4). The observed rate constant is given by Eq. (5), the sum of the rate constant for

$$CcP - I + ACN \overset{K_D}{\leftrightarrows} CcP - I - ACN \overset{k_{cat}}{\rightarrow} CcP + CEO \quad (4)$$

$$k_{obs} = k_{R1} + k_{cat}[ACN]/(K_D + [ACN]) \quad (5)$$

the endogenous reduction of compound I in the absence of acrylonitrile, k_{R1}, and the term for reduction of CcP compound I by acrylonitrile. Fitting the data in Fig. 4 to Eq. (5) with k_{R1} and K_D fixed at their independently determined values, $1.3 \times 10^{-3}\ s^{-1}$ and 1.1 M, respectively, gives a value of $0.0102 \pm 0.0030\ s^{-1}$ ($0.61 \pm 0.18\ min^{-1}$) for k_{cat}, within the range of the maximum observed microsomal cytochrome P-450 turnover rates for acrylonitrile epoxidation [26–31].

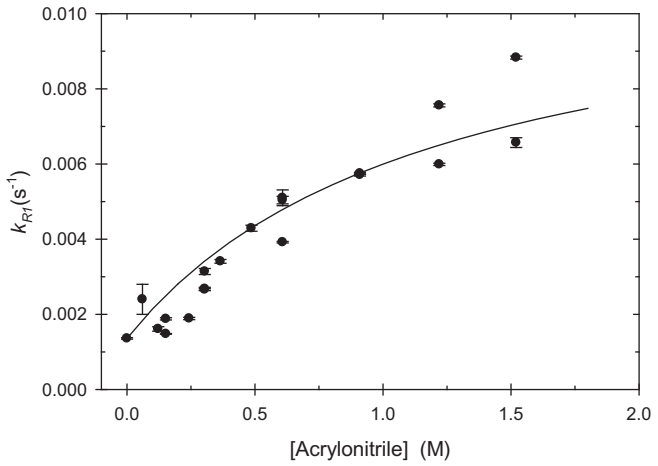

Fig. 4. The effect of acrylonitrile on the fast phase of CcP compound I reduction. The data were fit to Eq. (5) of the text using non-linear regression. The best-fit value for k_{cat} is $0.0102 \pm 0.003\ s^{-1}$ when k_{R1} and K_D are fixed at $1.3 \times 10^{-3}\ s^{-1}$ and 1.1 M, respectively.

4. Discussion

4.1. Binding of acrylonitrile to CcP

Although binding of isonitriles to the Fe(II) form of heme proteins is well known [1], we have been unable to find any reports of nitrile binding to heme proteins, either in the Fe(II) or Fe(III) state. There are several reports on the oxidation of acrylonitrile by cytochrome P450 indicating that acrylonitrile can bind within the heme pocket of cytochrome P450 although not necessarily as a heme ligand. Michaelis constants for acrylonitrile in the cytochrome P450-catalyzed monooxygenase reaction vary widely, from 52 μM [28] to 0.19 M [26]. In a search for small, neutral ligands without associated proton binding/dissociation chemistry to complicate interpretation of the pH dependence of ligand binding, we decided to try binding small organonitriles to CcP. We observed no interaction with acetonitrile but we did observed a weak association with acrylonitrile. The spectroscopic changes associated with the interaction, Fig. 1, are consistent with conversion of the five-coordinate, high-spin heme in CcP to a six-coordinate, low-spin complex. The rate constants and the equilibrium dissociation constants are consistent with a simple one-step binding reaction as described in Eq. (2). The reaction is independent of pH between 4 and 7.5 indicating that ionization of amino acid residues near the heme site do not affect the binding. Above pH 7.5, CcP begins to undergo a complex series of reactions in an alkaline transition that ultimately leads to denaturation of the enzyme [37]. The high concentrations of the organic ligand used to probe acrylonitrile binding at pH 8 appears to promote the alkaline transition leading to complex kinetics for binding of the ligand (additional material file) although the K_D value determined from equilibrium measurements at pH 8 are consistent with those determined at lower pH. Interestingly, CcP binds acrylonitrile with a higher affinity than it binds imidazole (JEE – unpublished observations).

4.2. Oxidation of acrylonitrile by CcP

Acrylonitrile is a widely used industrial chemical and numerous studies on its toxicology and metabolism have been undertaken [26–31]. A major detoxification pathway involves epoxidation of acrylonitrile by cytochrome P450 to 2-cyanoethylene oxide, followed by hydrolysis of the epoxide and generation of a wide variety of protein, nucleic acid, and glutathione adducts. Most kinetic studies have been done with microsomal preparations. The turnover rates vary over a very large range, from $1.3 \times 10^{-4}\ min^{-1}$ for cytochrome P450-2E1 [29] to $4.9\ min^{-1}$ for acetone-treated rat liver microsomal preparations [31].

Miller et al. [38] have shown that CcP and a CcP mutant, CcP(W51A), have low-levels of peroxygenase activity, catalyzing the epoxidation of styrene, *trans*-β-methylstyrene, and *cis*-β-methylstyrene by hydrogen peroxide. The turnover numbers are 0.012 and $0.017\ min^{-1}$ for the oxidation of *trans*-β-methylstyrene and *cis*-β-methylstyrene, respectively, about two orders of magnitude slower than the cytochrome P450-catalyzed oxidation of styrene. Since CcP has peroxygenase activity and since we have demonstrated that acrylonitrile can access the heme pocket in CcP, we decided to investigate oxidation of acrylonitrile by CcP compound I. The data in Fig. 3 demonstrate that CcP compound I oxidizes acrylonitrile. Extrapolating to infinite acrylonitrile concentration gives a maximum turnover number of $0.61\ min^{-1}$.

We have been unable to find any studies of acrylonitrile oxidation via the "peroxide shunt" pathway with cytochrome P50s but these rates tend to be slower than the monooxygenase rates for the same substrates. The maximum turnover rate for the CcP-catalyzed oxidation of acrylonitrile by hydrogen peroxide, $0.61\ min^{-1}$,

is well within the range of the monooxygenase rates reported for microsomal preparations [26–31].

We have demonstrated for the first time that acrylonitrile can bind to a ferric heme protein. The binding mechanism appears to be a simple, one-step association of the ligand with the heme iron, and in the case of CcP, the affinity of the enzyme for acrylonitrile is greater than that for imidazole. We have also demonstrated that CcP can catalyze the oxidation of acrylonitrile, most likely to 2-cyanoethylene oxide in a "peroxygenase" reaction, with rates that are similar to rat liver microsomal cytochrome P450-catalyzed oxidation in the monooxygenase reaction.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.11.084.

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