A Distinctive Electrocatalytic Response from the Cytochrome c Peroxidase of Nitrosomonas europaea*

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Here the cytochrome c peroxidase (CcP) from Nitrosomonas europaea is examined using the technique of catalytic protein film voltammetry. Submonolayers of the bacterial diheme enzyme at a pyrolytic graphite edge electrode give catalytic, reductive signals in the presence of the substrate hydrogen peroxide. The resulting wave-shapes indicate that CcP is bound non-covalently in a highly active configuration. The native enzyme has been shown to possess two heme groups of low and high potential (L and H, −260 and +450 mV versus hydrogen, respectively), and here we find that the catalytic waves of the N. europaea enzyme have a midpoint potential of >500 mV and a shape that corresponds to a 1-electron process. The signals increase in magnitude with hydrogen peroxide concentration, revealing Michaelis-Menten kinetics and $K_m = 55 \mu M$. The midpotential shifts with substrate concentration, indicating the electrochemically active species observed in our data corresponds to a catalytic species. The potentials also shift with respect to pH, and the pH dependence is interpreted in terms of a two pK_a model for proton binding. Together the data show that the electrochemistry of the N. europaea cytochrome c peroxidase is unlike other peroxidases studied to date, including other bacterial enzymes. This is discussed in terms of a catalytic model for the N. europaea enzyme and compared with other cytochrome c peroxidases.

This report describes the first demonstration of direct, catalytic electrochemistry of a diheme peroxidase using the technique of protein film voltammetry (PFV) (1–3). Bacterial cytochrome c peroxidases (CcP) are periplasmic enzymes found in bacteria, which carry out the 2-electron reduction of hydrogen peroxide (4). The characterized examples of bacterial peroxidases are all diheme enzymes, making them distinct from the superfamily of plant and yeast peroxidases that contain a single heme unit (5, 6). It has been proposed that the bacterial diheme enzymes store electron equivalents differentially than the monoheme-type CcPs; while the latter are known to generate Compound I in the course of catalysis (containing either a porphyrin radical or an oxidized Trp residue) (7), the bacterial enzymes appear to use a ferrous secondary heme of high potential (H) to store the same electron equivalent needed to generate the radical-based Compound I in monoheme CcPs (8). The bacterial CcP reaction cycle is depicted in Fig. 1, which shows that H formally cycles between the FeIII-H couple in the course of catalysis carried out at the low potential heme (L) active site (8). Fig. 1 also shows that the isolated enzyme (HIII-LIII) is unable to undergo efficient catalysis and first requires reduction by 1 electron to generate a highly active state: HIII-LIII. The reduction potentials of H and L are well separated, allowing the HIII-LIII form to exist as a stable state (e.g. +320 mV and −330 mV for the enzyme from Pseudomonas aeruginosa (9)). The scheme depicted in Fig. 1 requires a catalytic intermediate that is also HIII-LIII formally, the same oxidation state as the inactive isolated form of the enzyme, which indicates that the activation/deactivation process likely involves more than a single electron transfer step. As the CcP from P. aeruginosa (4), Paracoccus denitrificans (10), Rhodobacter capsulatus (11), and Pseudomonas stutzeri (12) all require reductive activation, we believe that bacterial cytochrome c peroxidases pose an interesting problem regarding the interplay between redox chemistry and enzymatic activity.

The CcP from Nitrosomonas europaea (NeCcP) also contains L and H hemes (−260 mV and +450 mV versus SHE) thought to act as the active site and an electronic conduit, respectively (13). However, NeCcP is unlike most other bacterial CcP enzymes, as NeCcP is fully active in the all oxidized, isolated form (13). This property is understood somewhat from consideration of the crystal structure of the enzyme, which possesses an open coordination site at LHIII (14). Conversely, the structure of the P. aeruginosa CcP (PaCcP) shows that the heme iron of L is coordinately saturated (15) in the fully oxidized and inactive state (Fig. 1). It has been proposed that reduction of H in PaCcP causes the loss of a ligand at L (15), based on EPR, Mössbauer, and optical studies (8). The conformational change induced by ligand loss is likely part of the mechanism of activation. This also explains why the enzyme does not deactivate when it reaches the HIII-LIII oxidation level as a part of the normal catalytic cycle; catalysis proceeds faster than the conformational reorientation associated with the inactive state. In comparison, the active site in the N. europaea enzyme appears ready to bind substrate in the HIII-LIII state. Thus, Fig. 1 may not apply for NeCcP, and H need not exist as a long lived, reduced species in an intermediate state of the catalytic mechanism, as is required for other bacterial CcP enzymes.

To investigate the link between redox chemistry and catalytic activity, we have initiated a study of the electrochemistry of the simpler of these enzymes, NeCcP, using the technique of PFV. PFV of redox active enzymes has proven to be a remarkably versatile and powerful tool to shed light upon the role of redox chemistry in enzyme mechanisms (1–3). PFV can directly

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† The abbreviations used are: PFV, protein film voltammetry; CcP, cytochrome c peroxidase; NeCcP, N. europaea CcP; PaCcP, P. aerugi-nosa CcP; PaGC, P. denitrificans CcP; yCcP, yeast CcP; PGE, pyrolytic graphite edge; MES, 2-N-morpholinoethanesulfonic acid; TAPS, N-tris-(hydroxymethyl)methyl-3-aminopropanesulfonic acid; CHES, (2-cyclohexylamino)ethanesulfonic acid.

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interrogate redox cofactors (e.g. hemes) and detect the catalytic chemistry of such cofactors when the experiment is carried out in the presence of substrate. In the case of bacterial Ccp enzymes, questions regarding the inter-cofactor electron transfer and the requirement for pre-reduction of H may be probed electrochemically. In this report, we show catalytic PFV that reports upon the active site of NeCcP and further distinguishes NeCcP from other members of the cytochrome c peroxidase superfamily. The results are interpreted in terms of a redox-based rationale explaining why NeCcP does not require prior pre-reduction of H.

MATERIALS AND METHODS

Enzymology—Samples of N. europaea cytochrome c peroxidase were prepared as reported previously (13). Aliquots of the 50 μg protein solution were stored at −80 °C between experiments.

Electrochemistry—All electrochemical experiments were carried out in a water-jacketed electrochemical cell positioned with a Faraday cage. A rotating disc working electrode of pyrolytic graphite edge (PGE) was used for all experiments, along with a platinum wire counter electrode, and saturated Calomel (Accumet) reference electrode. Working electrode rotation rate was controlled by an EG&G electrode rotator. Staircase cyclic voltammetry was carried out using a PGSTAT 12 Autolab (Ecochemie, The Netherlands) fitted with an ECD module for enhanced sensitivity. All potentials are reported with respect to the hydrogen electrode, and were corrected for electrode rotation rate above 2000 rpm. Inset A, a plot indicating the correlation between E_{cat} and the substrate concentration. E_{cat} varies with [H₂O₂] with a slope of ~62 mV per [H₂O₂] decade. Inset B, an Eadie-Hofstee plot of the catalytic response, taking the limiting current (i_{lim}) observed at 0.15 V as an enzymatic velocity; the negative slope of the plot equals K_m.

RESULTS AND DISCUSSION

We have used PFV to observe redox chemistry of NeCcP as immobilized in protein films that retain the natural properties of the enzyme. As shown in Fig. 2, protein films of NeCcP at a PGE electrode and in the presence of hydrogen peroxide yield catalytic electrochemical signals, which are observed as reversible, sigmoidal “waves.” Such waves display a limiting current (i_{lim}) that corresponds to an enzymatic velocity, and are centered at a potential (E_{cat}) that represents redox-linked chemistry within NeCcP. The observed electrochemical response is remarkably stable; multiple voltammetric cycles show minimal film loss and are highly reversible, indicating that protein denaturation does not occur in the course of measurements. We find that the magnitude of i_{lim} scales with peroxide concentration and eventually saturates, in accord with Michaelis-Menten kinetics. Also, i_{lim} is invariant with rotation rate above 2000 rpm and shows little decay over time, indicating that diffusion and mechanical stability of the protein films do not influence the magnitude of the catalytic response. Taking i_{lim} as an enzymatic velocity, a Michaelis-Menten treatment results in an Eadie-Hofstee plot (Fig. 2, inset B) that reveals values of K_m and k_cat, which are in general agreement with other values reported for other diheme peroxidases (12, 16); K_m is found to be 55 μM for H₂O₂ and k_cat is estimated to be over 1000 s⁻¹. We note that for our data, determination of a value of k_cat is achieved through an estimation of electroactive coverage, which is typically calculated through the peak area of non-turnover signals (1–3). In the absence of peroxide, non-turnover signals are not typically observed, due to low electroactive coverage, and therefore we estimate an electroactive coverage of <0.5 pmol/cm² (17) and estimate k_cat using this as an upper limit. We note that our estimated value is in agreement with literature values for other Ccp enzymes (10) and 300 times faster than the published value for NeCcP in an assay using horse heart cytochrome c as an electron donor for the less active, half-reduced enzyme (13). Additionally, voltammograms of bulk-phase solutions of NeCcP (data not shown) show weak signals, which correspond to the previously reported potentials of the enzyme: +450 mV for H^{III} and −260 mV for L^{III} (13). Together, these findings indicate that the NeCcP is not greatly perturbed by interacting with an electrode.

The data shown in Fig. 2 contrast sharply with previous PFV studies of peroxidases and immediately distinguishes NeCcP from other peroxidases studied by electrochemistry; Armstrong and co-workers have shown the monoheme yeast Ccp (yCcP) yields a single sharp non-turnover signal due to the reversible 2-electron generation of Compound I, at +750 mV (18, 19).
Complementing catalytic signals were also observed at the same potential and reveal cooperative electron transfer (18, 19). Furthermore, the bacterial P. denitrificans CcP (PdCcP) has been shown to give irreversible, non-turnover electrochemical signals in bulk phase cyclic voltammetry (20). The irreversibility of both the H and L signals was taken to indicate the redox-linked activation process required for the L active site as shown in Fig. 1; no catalytic activity was observed for PdCcP (20). In contrast to both of these cases, NeCcP films give reversible catalytic waveforms (Fig. 2) centered at a midpoint potential ($E_{cat}$) significantly less than what has been observed in analogous voltammetry of yeast CcP Compound I. The sigmoidal waveforms seen in Fig. 2 have a value of $E_{cat}$ of +515 mV or greater (see inset A), depending upon the substrate concentration, making it distinct from both (a) the resting $H^{III/I}$ potential (+450 mV) and (b) the potential reported 2-electron generation of Compound I (+750 mV) (18).

In the assignment of the catalytic wave to a specific redox process, we note that there are two principle possibilities: $E_{cat}$ may represent a rate-limiting electron transfer from the H center (as suggested for the Pseudomonas-type CcP, as shown in Fig. 1) or $E_{cat}$ may involve an intrinsic electron transfer-linked step of the catalytic site. Regarding the former possibility, the wave shape of the NeCcP catalytic response can be modeled in terms of an $n = 1$ process, and there is no evidence of cooperativity, unlike the yCcP findings of Armstrong and co-workers (18). Furthermore, the observed value of $E_{cat}$ is close to the $H^{III/I}$ couple and suggests that the observed catalytic voltammetry may be due to intramolecular electron transfer from H to the L active site as would be required for the mechanism shown in Fig. 1. Such a proposal would render the observed $K_{cat}$ a rate-limiting electron transfer constant, and $E_{cat}$ would be due to the $H^{III/I}$ couple. Yet, for NeCcP there is no indication of the electrochemical irreversibly observed for the Paracoccus enzyme which requires reduction of H to become active (20), and $E_{cat}$ is greater than the value of the $H^{III/I}$ potential. Regarding the issue of irreversibility and electrochemical activation, the catalytic waves we observe have highly reversible shape with respect to both the scan rate and the starting potential of the scan. Both observations indicate that $E_{cat}$ corresponds to a fast electrochemical event that would reveal the activation step required by other peroxidases.

Several additional pieces of evidence argue in favor of the assignment of $E_{cat}$ to an active-site redox process and against the possibility that $E_{cat}$ represents the electron transfer from H. First, $E_{cat}$ is dependent upon $H_2O_2$ with a gradient of $-62$ mV per $[H_2O_2]$ decade as shown in Fig. 2 (inset A). This indicates that $E_{cat}$ is associated with a catalytic feature dependent upon peroxide binding, a property that should be associated with the active site. Second, the pH dependence of $E_{cat}$ displays a complex relationship between $E_{cat}$ and pH. As shown in Fig. 3, $E_{cat}$ obeys a linear dependence at the extrema of pH, with a gradient of 64 mV/decade, yet appears to be approximately pH-independent at intermediate values. The observation of this complex pH dependence also reinforces our suggestion that $E_{cat}$ corresponds to a process occurring at the active site, as he labeled groups involved in simple electron transfer reactions rarely demonstrate a pH dependence. For example, the heme groups of the multiheme enzyme hydroxylamine oxidoreductase have been found to possess redox potentials that are independent from pH, except for the P460 active site, which displays a pH dependence of $-60$ mV/decade (21).

The data from Fig. 3 are readily fit to a two $pK_a$ model in which both the oxidized state and the reduced state can be protonated independently (Fig. 3) as described by Equation 1 (22, 23).

$$E = E^\circ + \frac{2.303RT}{nF}\log \left( \frac{[H^+][H^+]K_{cat}}{[H^+]} \right)$$

(Eq. 1)

This model yields $pK_a$ values of 6.5 $\pm$ 0.5 and 8.4 $\pm$ 0.2 for the reduced and oxidized species, respectively. The observation of the two $pK_a$ values is invariant with respect to $H_2O_2$ concentration used in the experiment. We take the pH dependence as further evidence that $E_{cat}$ represents an active site species and that $E_{cat}$ does not represent a mediating electron transfer process from the electrode. We note the observed $pK_a$ values are similar to those observed in a monoheme CcP (24), as well as those found for an active site histidine and bound Fe(III) aquo in studies of myoglobin (25). However, the source of the $pK_a$ values are not clear currently, while the superfamily of peroxidases typically contains a conserved Arg and His motif at the peroxide binding site; NeCcP contains an Gln and Glu residue (14). Thus, further experiments will be needed to determine the identity of the specific proton donors to the NeCcP active site.

The catalytic voltammetry observed above indicates a redox-active process at the NeCcP active site, which requires the binding of both protons and substrate. Given this, and the observation that NeCcP is fully active in the isolated form (12), we propose that the chemical identity of $E_{cat}$ is best understood in terms of a redox cycle of the enzyme shown in Fig. 4. This scheme suggests a mechanism that is much more like that of the monoheme CcP enzymes; substrate binds at L in an active $H^{III/I}$ state, and the 2-electron reduction of hydrogen peroxide generates Compound I through the oxidation of the L iron to the [FeIV=O] species and another residue, R, which results in a stable radical. (The nature of the R group shown in Fig. 4 is not clear currently and may be a porphyrin-based radical or another redox active group in analogy to the yCcP.) Compound II is subsequently generated and reduced in two independent $n = 1$ electron transfer steps. Here Compound II is formally a...
[FeV=O] species and not the diferric form of the enzyme involved in the mechanisms of other bacterial CcP enzymes, as shown in Fig. 1. Thus, for NeCcP H does not play the role observed for most other CcP (e.g. the *Pseudomonas* and *Paracoccus*) enzymes, where it effectively stores an electron for the catalytic chemistry occurring at L. Based on this premise, the highly reversible nature of the n = 1 voltammetric signals, the value of $E_{\text{cat}}$, the pH dependence of $E_{\text{cat}}$, and the lack of cooperativity observed in the catalytic wave shape all indicate that the electrochemical signal observed here represents the reduction of Compound II as depicted in Fig. 4, a formally proton-coupled electron transfer step.

Thus, NeCcP electrochemical catalysis appears to be controlled by two 1-electron events, in contrast to yCcP, which shows evidence of cooperativity. Furthermore NeCcP does not display any evidence of redox-linked activation that is required in other bacterial cytochrome c peroxidases. Our findings show that this can be understood in terms of the stepwise reduction of Compound I and Compound II that are a part of an enzymatic cycle in which the HIIILIII state is fully active (Fig. 4). We have also demonstrated that PFV will be a valuable tool for the study of the NeCcP mechanism; future experiments will focus upon the contribution of proton coupling in the chemistry of Compound II reduction.

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