A Single Histidine Is Required for Activity of Cytochrome c Peroxidase from Paracoccus denitrificans*

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The diheme cytochrome c peroxidase from Paracoccus denitrificans was modified with the histidine-specific reagent diethyl pyrocarbonate. At low excess of reagent, 1 mol of histidine was modified in the oxidized enzyme, and modification was associated with loss of the ability to form the active state. With time, the modification reversed, and the ability to form the active state was recovered. The agreement between the spectrophotometric measurement of histidine modification and radioactive incorporation using a radiolabeled reagent indicated little modification of other amino acids. However, the reversal of histidine modification observed spectrophotometrically was not matched by loss of radioactivity, and we propose a slow transfer of the ethoxyformyl group to an unidentified amino acid. The presence of CN⁻ bound to the active peroxidatic site of the enzyme led to complete protection of the essential histidine from modification.

Limited subtilisin treatment of the native enzyme followed by tryptic digestion of the C-terminal fragment (residues 251–338) showed that radioactivity was located in a peptide containing a single histidine at position 275. We propose that this conserved residue, in a highly conserved region, is central to the function of the active mixed-valence state.

Bacterial cytochrome c peroxidases (CCPs)1 are diheme enzymes that oxidize monoheme Class I cytochromes c and reduce hydrogen peroxide to water. The most extensively characterized example is from Pseudomonas aeruginosa and contains a high potential, electron-transferring heme and a low potential, peroxidatic heme (1, 2). In the oxidized state, the enzyme is inactive (1, 2); the high potential heme is partially high spin, and its low potential heme to become high spin and enables binding of hydrogen peroxide (4, 5). We have studied the enzyme from Paracoccus denitrificans and have found very similar characteristics (6, 7), although a distinctive difference is the Ca2⁺ requirement in P. denitrificans CCP for the low to high spin transition at the peroxidatic heme (8).

The amino acid sequence of P. aeruginosa CCP suggested that the molecule was constructed in two domains, each having the general appearance of a monoheme Class I cytochrome (9). This family of cytochromes is characterized by a heme attachment site and proximal histidine ligand near the N terminus and a distal methionine ligand near the C terminus. Ellfolk et al. (9) proposed that the C-terminal domain followed this pattern and acted as the high potential electron-transferring site. The N-terminal domain appeared to lack an appropriately placed methionine, and Ellfolk et al. proposed that the sixth iron ligand of this domain was a histidine donated from the C-terminal domain and conferring a low redox potential. In the mixed-valence form of the enzyme, this histidine would be released from the iron, the heme would become high spin, and hydrogen peroxide could enter the active site.

The amino acid sequence of P. aeruginosa CCP has been revised on the basis of the gene sequence determined by Ridout et al. (10), and the key histidine in the proposal of Ellfolk et al. (9) is His-261. We have determined the amino acid sequence of P. denitrificans CCP,2 which shows 61% identity to the P. aeruginosa enzyme. The histidine in P. denitrificans CCP corresponding to His-261 in P. aeruginosa CCP is at position 275.

During the preparation of this paper, the crystallographic structure of the oxidized form of the P. aeruginosa enzyme was determined (11). In contrast to the original proposal of Ellfolk et al. (9), the ligand to the proposed N-terminal peroxidatic heme is, in fact, His-71 (position 85 in P. denitrificans CCP numbering), and His-261 is situated at the interface of the two heme domains.

This paper describes the modification of P. denitrificans CCP with the histidine-selective reagent diethyl pyrocarbonate. We show that modification of His-275 in the oxidized form of the enzyme completely abolishes the ability of the enzyme to form the active mixed-valence state.

EXPERIMENTAL PROCEDURES

Enzyme Purification—P. denitrificans (Laboratory of Microbiology, Depts.) was grown and cytochrome c peroxidase was purified as described by Goodhew et al. (12). After purification, the protein displayed an A_{408nm}/A_{280nm} ratio of ~5 and gave a single band on SDS-polyacrylamide gel electrophoresis. Concentrated stocks of the protein in 5 mM Mes, pH 6, 10 mM NaCl were stored at ~40 °C. The concentration of P. denitrificans CCP was determined at 408 nm using ε = 250 M⁻¹ cm⁻¹.

1 The abbreviations used are: CCPs, cytochrome c peroxidases; Mes, 2-(N-morpholino)ethanesulfonic acid; DEPC, diethyl pyrocarbonate; HPLC, high performance liquid chromatography.

2 J. Van Beeumen and G. W. Pettigrew, unpublished results.
The enzyme was purified in the oxidized state. The mixed-valence low spin Sigma and used without further purification.

The mixed-valence enzyme after Ca\(^{2+}\) gave rise to the mixed-valence high spin state (6). The ability of the sample to form the latter state was measured by difference spectra of the mixed-valence enzyme after Ca\(^{2+}\) treatment minus those of the mixed-valence enzyme.

Diethyl Pyrocarbonate—Both DEPC and [carbonyl-\(^{14}\)C]DEPC were purchased from Sigma and stored desiccated at 4 °C. Stock solutions were made by dilution of DEPC into acetonitrile and assayed by reaction with 5 mM imidazole, pH 7.5 (14). Horseradish peroxidase, in the presence of added Ca\(^{2+}\), was purified by the method of Pettigrew and Selman (13). The concentration of yeast cytochrome c peroxidase was determined at 408 nm using \(e = 95 \text{ M}^{-1} \text{ cm}^{-1}\) (14). Horse heart cytochrome c (type VI) was purchased from Sigma and used without further purification.

Formation of the Different Redox States of Cytochrome c Peroxidase—The enzyme was purified in the oxidized state. The mixed-valence low spin state was formed in the presence of 1 mM Ca\(^{2+}\), 10 \(\mu\)M diaminoduril (6). The same reductive treatment in the presence of 3 mM Ca\(^{2+}\) gave rise to the mixed-valence high spin state (6). The ability of the sample to form the latter state was measured by difference spectra of the mixed-valence enzyme after Ca\(^{2+}\) treatment minus those of the mixed-valence enzyme.

Assay of Cytochrome c Peroxidase—The activity of mixed-valence cytochrome c peroxidase, in the presence of added Ca\(^{2+}\), was measured by following the decrease in absorbance of the \(\alpha\)-band of ferrocytochrome c as it was oxidized (7). Assays were initiated by addition of the enzyme (1 \(\mu\)M) to a cuvette containing 5 mM Hapes, pH 7.5, 10 mM NaCl, 7 \(\mu\)M horse heart ferrocytochrome c, 1 mM Ca\(^{2+}\), and 18 \(\mu\)M hydrogen peroxide. The percent activity of each sample in Fig. 2b was calculated on the basis of their respective turnover numbers, with the unmodified preactivated control turnover number representing 100%. Turnover numbers were calculated using the initial gradient of cytochrome c oxidation (7).

Spectroscopy—All spectra in the UV region were recorded on a Cary 219 spectrophotometer. Difference spectroscopy in the visible region was performed with a single beam Philips PU8700 spectrophotometer. Spectra were collected at 23 °C using 1-cm path length quartz cuvettes. EPR measurements were performed using a Bruker ESP 380 spectrometer with an Oxford Instruments ESR-9 continuous flow cryostat. The relative intensities of the EPR signals were determined using software that simulates the experimentally determined spectra using the values for \(g_{\text{iso}}, g_{\text{tor}},\) and \(g_{93}\) and integrates the resultant signals (17).

Peptide Separation and Mass Spectrometry—The Glu-1–Thr-250 and Arg-251–Met-338 peptides were purified as described in the legend to Fig. 7. They were desalted by passage through a Superdex 75 column (Pharmacia Biotech Inc.), equilibrated in 0.1 \(\text{M}\) ammonia, and lyophilized. The tryptic peptides of Arg-251–Met-338 were separated by HPLC on a reverse-phase Tephosphere 50 DS column (25 cm \(\times\) 4.6 mm) using a gradient of 0–70% acetonitrile in 0.1% trifluoroacetic acid. The column eluate was monitored at 214 and 280 nm.

Mass spectrometry was performed using a BioQ triple quadrupole mass spectrometer equipped with a pneumatically assisted electrospray source (Fisons, Altrincham, United Kingdom) operating in positive ion mode. The resolution of the mass spectrometer allows for isotopic resolution of peptides under M, \(\sim 3000\). By averaging the mass of elemental isotopes according to their natural distribution and thus determining the average mass of individual residues, the theoretical M, values of peptides over 3000 were calculated.

Secondary Structure Predictions—We used the secondary structure prediction methods of Gibrat et al. (18), Levin et al. (19), Deleage and Roux (20), and Geourjon and Deleage (21) contained in the package “SOPMA.”

RESULTS

Extent of Modification of P. denitrificans CCP by Diethyl Pyrocarbonate—Diethyl pyrocarbonate reacts with both free imidazole in solution and histidyl residues in proteins, yielding the ethoxyformyl derivatives. The reaction may be followed spectrophotometrically by a gain of absorbance between 230 and 250 nm. Fig. 1 compares difference spectra (230–300 nm) of the modification of P. denitrificans CCP (at 2 and 20 min), imidazole, and yeast CCP.

The difference spectrum of the modified P. denitrificans CCP displays an initial gain of absorbance at 245 nm (Fig. 1A, panel i), followed by slow formation of a trough between 265 and 300 nm (panel ii). Modification of free imidazole generated the ethoxyformylimidazole derivative, which gave a peak maximum at 233 nm, but no corresponding changes at higher wavelengths (Fig. 1B). As will be discussed below, the loss of ability to form the active state of P. denitrificans CCP is correlated with the formation of the ethoxyformylimidazole derivative.

Diethyl pyrocarbonate was incubated with P. denitrificans CCP (3.5 \(\mu\)M) in 50 mM Hapes, pH 7.5, was modified with DEPC to a final concentration of 20 \(\mu\)M. After 2 min, a modified CCP minus unmodified CCP difference spectrum was taken and showed 0.57 mol of histidine modified per mol of protein, based on \(e_{245\text{nm}} = 3.2 \text{ M}^{-1} \text{ cm}^{-1}\) for ethoxyformylimidazole. Panel ii, the reaction of DEPC with P. denitrificans CCP was complete within 20 min and showed 1.0 mol of histidine modified per mol of protein. B: 5 mM imidazole in 50 mM Hapes, pH 7.5, was incubated with 4 \(\mu\)M DEPC. The difference spectrum obtained at 2 min showed 3.8 \(\mu\)M ethoxyformylimidazole (\(e_{245\text{nm}} = 3.0 \text{ M}^{-1} \text{ cm}^{-1}\)). The spectrum did not change in the subsequent 20 min. C: yeast CCP (3.8 \(\mu\)M) was incubated with 4 \(\mu\)M DEPC. The difference spectrum was corrected to a final concentration of 20 \(\mu\)M. After 15 min, a difference spectrum was taken and showed 1.1 mol of histidine modified per mol of protein. The wavelengths of individual peak maxima are indicated on the respective spectra.

FIG. 1. UV difference spectra of the modification of imidazole, P. denitrificans CCP, and yeast CCP with diethyl pyrocarbonate. A, panel i, P. denitrificans CCP (3.5 \(\mu\)M) in 50 mM Hapes, pH 7.5, was modified with DEPC to a final concentration of 20 \(\mu\)M. After 2 min, a modified CCP minus unmodified CCP difference spectrum was taken and showed 0.57 mol of histidine modified per mol of protein, based on \(e_{245\text{nm}} = 3.2 \text{ M}^{-1} \text{ cm}^{-1}\) for ethoxyformylimidazole. Panel ii, the reaction of DEPC with P. denitrificans CCP was complete within 20 min and showed 1.0 mol of histidine modified per mol of protein. B: 5 mM imidazole in 50 mM Hapes, pH 7.5, was incubated with 4 \(\mu\)M DEPC. The difference spectrum obtained at 2 min showed 3.8 \(\mu\)M ethoxyformylimidazole (\(e_{245\text{nm}} = 3.0 \text{ M}^{-1} \text{ cm}^{-1}\)). The spectrum did not change in the subsequent 20 min. C: yeast CCP (3.8 \(\mu\)M) was incubated with 4 \(\mu\)M DEPC. The difference spectrum was corrected to a final concentration of 20 \(\mu\)M. After 15 min, a difference spectrum was taken and showed 1.1 mol of histidine modified per mol of protein. The wavelengths of individual peak maxima are indicated on the respective spectra.
with absorbance gain at 245 nm and not with the presence of the 265–300 nm features.

The modified yeast CCP difference spectrum (Fig. 1C) is very similar to the modified P. denitrificans CCP spectrum. For both proteins, a DEPC/protein ratio of 20 μM:4 μM resulted in modification of ~1 mol of histidine/mol of protein. This was calculated using the extinction coefficient of ethoxyformylhistidine at 245 nm of 3.2 mM⁻¹ cm⁻¹ (16). A possible source of error for this calculation is the effect of spectral features near the peak at 245 nm. For example, the trough at 265–300 nm in the modified difference spectra of bacterial and yeast CCPs may have a "pulling down" effect on the 245 nm peak. An independent check on the extent of modification was made by measuring the incorporation of [14C]DEPC. These experiments have determined that, in general, there is good agreement between the two methods of quantitation, with radioactive labeling giving slightly less (~10%) than spectroscopic measurements. This indicates first that ΔA_{245 nm} is a reliable measurement of histidine modification and second that there is little uptake into amino acids other than histidine.

Fig. 2a shows the extent of modification of P. denitrificans CCP as DEPC concentration was raised. At each DEPC concentration, the modification was allowed to proceed until the absorbance maximum at 245 nm was obtained (20 min). The biphasic character of the plot of DEPC demonstrates the existence of an easily modifiable histidine. Eventually, at high DEPC concentrations, an apparent maximum of four histidines are modified (data not shown). We expected a maximum of three histidines to be available for modification since the remaining three are assumed to be coordinated to heme (two as proximal ligands and one as a distal ligand for the peroxidatic heme). The discrepancy that we see in our measured maximum value of four may be due to bis-modification of histidine (22). A similar biphasic pattern was obtained by following the extent of modification at a single high DEPC concentration (data not shown). A rigorous interpretation of the results was complicated by the fact that the final extent of modification is the result of the rate of modification, the rate of ethoxyformylhistidine reversal, and the rate of hydrolysis of the reagent. Fig. 2b compares the extent of modification at pH 6 and 7.5, and it indicates that much higher DEPC concentrations are required at pH 6 to achieve modification of 1 mol of histidine, a result consistent with the requirement that histidine must be unprotonated to be modified (23, 24). We chose pH 7.5 for subsequent studies.

The Most Easily Modified Histidine Is Essential for Activity—It is worth re-emphasizing that the active form of the enzyme is achieved by reduction of the electron-transferring heme and the Ca²⁺-dependent appearance of the high spin state at the peroxidatic heme. Thus, to assess the effect of modification on the oxidized enzyme, the protein must be reduced with ascorbate, and the appearance of either activity or the high spin band at 380 nm must be monitored on addition of Ca²⁺.

Fig. 3a demonstrates the loss of ability to form the high spin 380 nm band in the mixed-valence enzyme after modification of histidine in the oxidized state. These data are plotted in Fig. 3b along with loss of catalytic activity upon modification of histidine. Thus, modification of a single histidine in P. denitrificans CCP abolishes both the ability to form the high spin state and activity. Although a single histidine was also readily modified in yeast CCP at similarly low DEPC/protein ratios, modification did not cause loss of activity.

A further spectroscopic indicator of the active enzyme is the Ca²⁺-dependent appearance of a g_{max} = 2.89 signal in the EPR spectrum of the mixed-valence enzyme, which can be equated with the high spin peroxidatic heme at room temperature (Fig. 4a). Because of the high protein concentrations needed for EPR spectroscopy, endogenous Ca²⁺ allows partial appearance of this g_{max} = 2.89 signal in the spectrum of the mixed-valence without added Ca²⁺. The enzyme modified at a single histidine cannot undergo the transition to the active form (Fig. 4b) and is mostly trapped in a mixed-valence low spin form with g_{max} = 3.00.

Our difference spectrum due to modification at pH 7.5 showed the presence of a shallow trough between 265 and 300 nm (Fig. 1A, panel ii). We wanted to be certain that this was not an indicator of modification of aromatic acids such as tryptophan, which may affect the activity of the protein. A difference spectrum (Fig. 1A, panel i) obtained after modification for 2 min showed no trough between 265 and 300 nm, and yet the histidine modified correlated well with the proportion of protein unable to form the high spin state (data not

![Graph](http://www.jbc.org/)

**Fig. 2.** Extent of modification of P. denitrificans CCP with increasing concentrations of DEPC. a, P. denitrificans CCP (4 μM) in 5 mM Hepes, pH 7.5, was treated with increasing concentrations of DEPC, and the reactions were allowed to proceed to completion at each concentration. The moles histidine modified were calculated using ε_{245 nm} = 3.2 mM⁻¹ cm⁻¹. At a concentration of 8 mM DEPC, an apparent maximum of four histidines were modified (data not shown on figure). The boxed area indicates a subset of points used in b, b shows a comparison of histidine modification of CCP at pH 6 and 7.5. Modification of CCP (4 μM) at pH 6 was carried out in 5 mM Mes.

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**Fig. 3.**
shown). Also, during reversal of modification, the 245 nm peak is gradually lost, but the 265–300 nm trough remains (Fig. 5a). During reversal of modification, the loss of histidine modification correlates well with the recovery of the ability to form the active high spin state (Fig. 5b). The reversal of modification does not follow a simple exponential; in 5 mM Hepes, pH 7.5, 50% of the label is lost in 6 h. Taken together, these results indicate that the appearance of the 280 nm trough is not associated with changes in the activity of the enzyme and that those changes we observed are due to histidine modification.

Modification of the Different Redox States of P. denitrificans CCP and the Protective Effect of CN

The susceptibilities to modification of the three redox states of the enzyme are very different and not as we originally predicted. Fig. 6 shows that the oxidized enzyme at 4 μM loses all ability to form the active spin state at 20 μM DEPC, while this only occurs with the mixed-valence low spin and mixed-valence high spin states at higher concentrations of reagent. For the two mixed-valence forms of P. denitrificans CCP, histidine modification cannot be measured spectroscopically because ascorbate interferes with UV measurements. [14C]DEPC was used to determine the extent of modification in the oxidized and mixed-valence states (Fig. 6). The results indicate that the histidine modified in the oxidized enzyme at low levels of DEPC is not modified in the mixed-valence low spin state until higher levels of reagent are used. Thus, the essential histidine is more susceptible to modification in the oxidized state than in the mixed-valence low spin state. Similarly, the essential histidine is more susceptible to modification in the mixed-valence low spin state of the enzyme than in the mixed-valence high spin state. The continuation of the experiment in Fig. 6 shows that modification of the CN⁻ adduct of the mixed-valence high spin enzyme, with levels of DEPC that completely inactivate the same state in the absence of CN⁻, yields an enzyme that retains almost all activity. When CN⁻ is removed by molecular exclusion (a process that also reoxidizes the enzyme), the modified protein, at 4 μM,
can be further modified with a $5 \times$ excess of DEPC to give complete loss of the ability to form the high spin state. Addition of CN$^-$ to the oxidized and mixed-valence low spin states has no effect on the modifiability of the enzyme. These results show that an essential histidine is much less susceptible to modification when CN$^-$ is bound at the peroxidatic heme.

Identification of the Site of Modification in the Amino Acid Sequence—Subtilisin digestion of the native mixed-valence holoenzyme released two fragments that could be separated by molecular exclusion after dissociation in SDS (Fig. 7). SDS-polyacrylamide gel electrophoresis analysis of the elution profile of the molecular exclusion column showed complete resolution of the larger from the smaller peptide (Fig. 7). However, the remaining undigested whole protein, which accounted for 7% of the total protein by densitometry of the Coomassie Blue-stained gel, coeluted with the larger peptide. Although the level of undigested protein could be lowered by more intense digestion, this tended to increase the amount of fragments of the larger peptide that coeluted with the smaller peptide (data not shown).

Mass spectrometry established that the larger fragment ($M_r 27,950.9$) corresponded to the N-terminal 250 amino acids containing both heme groups ($M_r$ predicted from the amino acid sequence of 27,950.93). The smaller fragment ($M_r 9581.38$) accounted for the remaining non-heme C-terminal 87 amino acids ($M_r$ predicted from the amino acid sequence of 9579.87). Thus, subtilisin cleavage was limited to the peptide bond between Thr-250 and Arg-251.

Subtilisin digestion of the mixed-valence holoenzyme that had been radiolabeled in the oxidized state to the extent of 1 mol of histidine/mol gave the same peptide pattern as shown in Fig. 7. A ratio of $2.2:1$ for radioactive disintegrations in the Arg-251-Met-338 peptide relative to the Glu-1–Thr-250 peptide was obtained, and if the contribution of undigested protein to the Glu-1–Thr-250 fraction was allowed for, this ratio became $2.9:1$. This ratio is a strong indication that the single essential histidine resides in the Arg-251-Met-338 fragment. However, the close correlation between modification of a single histidine and loss of activity shown in Fig. 3 had led us to expect an even cleaner pattern of label distribution. We found that a $[^{14}C]$DEPC-labeled protein sample left for 24 h after modification had completely lost its ethoxyformylhistidine based on spectroscopic measurements at 245 nm, yet had retained almost all radioactivity. Cleavage and separation of the two subtilisin fragments yielded an Arg-251-Met-338/Glu-1–Thr-250 radiolabel ratio of $1.5$. Therefore, over the 24 h, label was transferred from a specific site on the Arg-251-Met-338 peptide to the Glu-1–Thr-250 peptide. We propose that a partial transfer of this type is the reason for the ratio of $2.9:1$ (rather than higher) that we observed for analysis completed within $-$2 h.

As the Arg-251-Met-338 peptide contains both His-275 and His-322, unequivocal identification of the essential histidine in CCP required isolation of a fragment containing a single hist-
Modified peptide was isolated by reverse-phase HPLC and identified by mass spectrometry. Therefore, the Arg-251–Met-338 peptide was subdigested with trypsin, generating six smaller fragments (labeled A–F) (Fig. 8) that were isolated by reverse-phase HPLC and identified by mass spectrometry. The essential histidine, which, when ethoxyformylated, increases the retention time of the peptide, was confirmed by obtaining an elution profile that matched that of the unmodified protein after reversal of the modification in 0.2 M ammonia solution (data not shown). Also, the M_r of peptide E2 was determined to be 2472.62 by mass spectrometry. This corresponds well with the theoretical mass of 2400.12 for peptide E (Asn-266–Lys-287) plus 72 for an ethoxyformyl group. Thus, His-275 is the single histidine modified at low DEPC/CCP ratios that is essential for activity.

**DISCUSSION**

Modification of a Single Essential Histidine—Spectrophotometric and radioactive labeling measurements are consistent with the modification of a single histidine at low DEPC concentrations. Although the trough between 265 and 300 nm resembles that expected for O-ethoxyformylation of tyrosine (25), the extent of the absorbance change would imply a modification of at least 1 mol of tyrosine/mol of protein (ε278 nm = 1.3 × 10^3 M^-1 cm^-1), and this is certainly not supported by measurement of radiolabeling. A similar spectroscopic feature is present in modified yeast CCP and other proteins modified with DEPC (25, 26).

Modification of a single most reactive histidine is associated with loss of activity (Fig. 3), and activity is regained as the modification slowly hydrolyzes (Fig. 5). In yeast CCP, 1 mol of

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**Fig. 7. Identification of the Arg-251-Met-338 peptide as the site of modification.** CCP (20 μM) in 10 mM Hepes, pH 7.5, was modified to an extent of 0.9 mol of histidine modified per mol of protein with [14C]DEPC (100 μM). The modified sample was desalted through a small Sephadex G-25 column (22 × 1 cm), equilibrated in 10 mM Hepes, pH 7.5, before being digested with subtilisin. To CCP (4–20 μM) in 10 mM Hepes, pH 7.5, were added 1 mM ascorbate, 10 μM diamino dural, and 1 mM CaCl_2. Subtilisin (Carlsberg) was added to give a substrate/ enzyme ratio of ~50:1 (w/w). After 60 min at 0°C, subtilisin was inhibited by 1 mM phenylmethanesulfonyl fluoride, 10 mM EGTA. The resultant peptides were separated by HPLC as described under "Experimental Procedures" and gave rise to the two main peaks labeled Unmodified. The unmodified purified Arg-251–Met-338 peptide was prepared as described in the legend of Fig. 7. Trypsin was added to give a substrate/enzyme ratio of 50:1 (w/w). After 10 min at 23°C, trypsin was inhibited with 0.5 mM phenylmethanesulfonyl fluoride. The resultant peptides were separated by HPLC as described under "Experimental Procedures" and gave rise to the two main peaks labeled Unmodified. The unmodified peptide was purified by reverse-phase HPLC and identified by mass spectrometry. The modified peptide was isolated by reverse-phase HPLC and identified by mass spectrometry. The essential histidine, which, when ethoxyformylated, increases the retention time of the peptide, was confirmed by obtaining an elution profile that matched that of the unmodified protein after reversal of the modification in 0.2 M ammonia solution (data not shown). Also, the M_r of peptide E2 was determined to be 2472.62 by mass spectrometry. This corresponds well with the theoretical mass of 2400.12 for peptide E (Asn-266–Lys-287) plus 72 for an ethoxyformyl group. Thus, His-275 is the single histidine modified at low DEPC/CCP ratios that is essential for activity.
histidine is also readily modifiable at low concentrations of DEPC, but in this case, modification was not associated with loss of activity (data not shown). This is consistent with the results of Bosshard et al. (27), who found that three histidines could be modified with no loss of activity. Similarly, in horse-radish peroxidase, two histidines were modified by DEPC, but it is the more slowly modified residue that is essential (26).

Identification of the Essential Histidine as His-275—If we assume that two histidines that remain unmodified at high DEPC concentrations are the conserved proximal ligands to the two heme groups, His-85 and His-275 are the only remaining conserved histidines, and therefore, one or the other (or both) is likely to be essential for activity. Because of the relatively rapid loss of the ethoxyformyl group, few studies have attempted location of a modified histidine in a peptide digest, although there are some exceptions (28). In our case, there was the additional problem that c-type cytochromes tend to be resistant to proteolysis unless the heme is removed, but this procedure requires conditions (0.1 M HCl, 8 M urea) that would likely to accelerate loss of ethoxyformyl groups. However, we were able to exploit a remarkable single bond cleavage by subtilisin in the native state, which releases a C-terminal fragment containing the conserved His-275 and one other nonconserved histidine and an N-terminal fragment containing the conserved His-85 and one other nonconserved histidine. This cleavage between Thr-250 and Arg-251 does not reflect limitations in the action of subtilisin, but rather the limited access of protease to the native substrate.

Purification of the C-terminal fragment by molecular exclusion chromatography followed by tryptic digestion and peptide separation by reverse-phase HPLC allowed localization of radioactive labeling in the tryptic peptide containing His-275 within 4 h of modification. Over a longer time scale (e.g., 24 h), the radioactive label was retained on the protein, but attached to the N-terminal region (Glu-1–Thr-250). We are unaware of a precedent for such an intramolecular transfer of an ethoxyformyl group, but we propose that it is consistent with attack on ethoxyformyllhistidine 275 by a lysine that is adjacent in the folded protein, but resides in the N-terminal sequence (Glu-1–Thr-250). Ethoxyformyllysine is not susceptible to nucleophilic attack (15). This proposal will be tested by identification of the labeled residue in the Glu-1–Thr-250 peptide.

Role of His-275—We have shown that His-275 is essential for activity. Consistent with this is its position in a highly conserved region of the cytochrome c peroxidase molecule. Ridout et al. (10) have corrected parts of the P. aeruginosa CCP sequence, and in this region (P. denitrificans Asp-255–Trp-280 and P. aeruginosa Asp-241–Trp-266), 25 out of 26 residues are identical. 4

In the crystallographic structure of the P. aeruginosa enzyme (11), His-261, the counterpart of His-275, is placed at the interface between the N-terminal domain (proposed to contain the peroxidatic heme) and the C-terminal domain (containing the electron-transfer heme). Fig. 7 of Ref. 11 shows the histidine as part of a hydrogen-bonded network involving the juxtaposed heme propionates of both heme groups. Our finding that His-275 is the most easily modified histidine in the oxidized state would not have been consistent with the original role as heme ligand proposed by Elfolfk et al. (9), but it is compatible with the position determined by x-ray crystallography.

Fulop et al. (11) proposed that this histidine may form part of an electron transfer route between the two heme groups. Our finding that its modification abolishes activity would be consistent with disruption of this electron transfer route. However, Fulop et al. realized that proposals concerning the active mixed-valence enzyme but based on the oxidized form must be regarded with skepticism. There are none of the likely catalytic residues near the proposed peroxidatic heme that one would expect by analogy with eukaryotic active sites (29, 30), and histidine 71 must leave the heme before a hydrogen peroxide-binding site can be formed. It is therefore entirely possible that major conformational changes take place during formation of the active mixed-valence enzyme. Indeed, one possibility is that the conserved region containing Arg-265, His-275, and Trp-280 moves to form the distal side of a restructured peroxidatic site. Although this sequence is not α-helical in the x-ray structure of the oxidized form (11), the consensus of the four prediction methods used revealed some α-helix in this region. For example, the method of Gibrat et al. (18) predicts α-helix between Asp-255 and Ala-268 in the P. denitrificans CCP sequence. In response to the criticism that such major conformational changes would be likely to slow, it must be realized that the oxidized form that has been crystallized is almost certainly not part of the catalytic cycle and represents a dead-end conformation into which the protein relaxes to protect the catalytic site in the absence of reductant.

Susceptibility of His-275 to Modification in Different Redox States—The much lower susceptibility to modification in the mixed-valence states may be due to a changed environment of His-275 that affects either its accessibility to the reagent or its protonation state. The presence of CN− bound at the peroxidatic heme further protects His-275 from modification. Again, this may be due to an effect on the protonation state of the essential histidine. Such an effect has been demonstrated in Arthromyces ramosus peroxidase (31) by showing the presence of a hydrogen bond donated by the distal His-56 to CN− bound to the iron. This suggests that CN− binding raises the pK of the histidine and would have the effect of making it less reactive with DEPC (23, 24). Alternatively, CN− may lock the protein in a conformation corresponding to the active state and with a concealed His-275, whereas in the absence of CN−, the mixed-valence protein is known to be in a temperature-dependent, high spin-low spin equilibrium (6).

Model for the Active Mixed-valence Form of the Enzyme—Because of the likelihood of substantial conformational change accompanying reduction of the enzyme, we will only have a reliable picture of the active site and the electron transfer routes when the crystallographic structure of the mixed-valence enzyme has been solved. The simplest model of the mixed-valence enzyme would be one in which His-71 (position 85 in P. denitrificans CCP) has dissociated from the N-terminal peroxidatic heme, but remains close by as a distal catalytic ligand. Another possibility alluded to above is that His-261 (position 275 in P. denitrificans CCP) and its accompanying conserved sequence move to form the distal environment of the N-terminal peroxidatic heme. Such a form would resemble the active enzyme proposed by Elfolfk et al. (9). We should keep our minds open to a third intriguing possible arrangement, and that is that His-261 and accompanying residues move to form the distal side of the C-terminal heme and that that heme becomes the peroxidatic center. In this heme switch model, the N-terminal heme would be ligated by the conserved Met-115 (position 129 in P. denitrificans CCP) and would act as the storage point for the electron. We have already described the possibility of such a mixed-valence state (32).

REFERENCES

Footnotes
4 G. W. Pettigrew and J. Van Beeumen, unpublished results.
A Single Histidine Is Required for Activity of Cytochrome c Peroxidase from *Paracoccus denitrificans*

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