The 1.4-Å crystal structure of the oxidized state of a Y25S variant of cytochrome cd₃ nitrite reductase from Paracoccus pantotrophus is described. It shows that loss of Tyr²⁵, a ligand via its hydroxy group to the iron of the cd₃ heme in the oxidized (as prepared) wild-type enzyme, does not result in a switch at the χ heme of the unusual bishistidinyl coordination to the histidine/methionine coordination seen in other conformations of the enzyme. The Ser²⁵ side chain is seen in two positions in the cd₃ heme pocket with relative occupancies of ~7:3, but in neither case is the hydroxy group bound to the iron atom; instead, a sulfate ion from the crystallization solution is bound between the Ser²⁵ side chain and the heme iron. Unlike the wild-type enzyme, the Y25S mutant is active as a reductase toward nitrite, oxygen, and hydroxylamine without a reductive activation step. It is concluded that Tyr²⁵ is not essential for catalysis of reduction of any substrate, but that the requirement for activation by reduction of the wild-type enzyme is related to a requirement to drive the dissociation of this residue from the active site. The Y25S protein retains the cd₃ heme less well than the wild-type protein, suggesting that the tyrosine residue has a role in stabilizing the binding of this cofactor.

Cytochrome cd₃ nitrite reductase is a dimeric enzyme of the bacterial periplasm; it plays a key role in denitrification, the respiratory reduction of nitrate to nitrogen gas. The cytochrome periplasm; it plays a key role in denitrification, the respiratory reduction of nitrate to nitrogen gas. The cytochrome cd₃ nitrite reductase is a dimeric enzyme of the bacterial periplasm; it plays a key role in denitrification, the respiratory reduction of nitrate to nitrogen gas. The cytochrome cd₃ nitrite reductase is a dimeric enzyme of the bacterial periplasm; it plays a key role in denitrification, the respiratory reduction of nitrate to nitrogen gas.

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feature of the oxidized cytochrome \( cd_1 \) from \( P. \) \textit{aeruginosa} \( (8) \) showed that the \( d_1 \) heme was ligated differently from that in \( P. \) \textit{pantotrophus}. In the former, a hydroxide was the distal ligand to the \( d_1 \) heme iron, but with a hydrogen bond to a tyrosine residue (position 10) that was in no sense equivalent in the structure to tyrosine 25 in the \( P. \) \textit{pantotrophus} enzyme. Tyrosine 10, which is not an essential residue \( (11) \), is not provided by the same subunit as that in which it is positioned close to the \( d_1 \) heme iron, \( i.e. \) there is a domain swapping \( (8) \). The c heme also differed, with the equivalent of methionine 106 of the \( P. \) \textit{pantotrophus} enzyme being a ligand in the oxidized \( P. \) \textit{aeruginosa} enzyme, along with the histidine of the CXXCH motif that is characteristic of a c-type cytochrome. The coordination of the c-type cytochrome heme in the oxidized \( P. \) \textit{aeruginosa} enzyme is thus equivalent to that in the reduced \( P. \) \textit{pantotrophus} enzyme.

In solution, spectroscopic analysis is consistent with ligation of tyrosine to the \( d_1 \) heme iron of the oxidized (as prepared) \( P. \) \textit{pantotrophus} enzyme and has demonstrated the same bishistidinyl coordination of the c heme as seen in the crystal \( (12) \). However, it has recently been shown that a catalytically more active form of the protein is obtained if the enzyme is first reduced and then oxidized by one of its alternative substrates, hydroxylamine \( (13) \). This much more active (initially) oxidized form has His/Met coordination at the c heme \( (14) \), while visible absorption and EPR spectra indicate that tyrosine 25 is not bound to the \( d_1 \) heme. Simple pre-reduction of the enzyme has also resulted in much higher \( k_{cat} \) values than could be obtained with the oxidized (as prepared) enzyme for any of the electron donor proteins: cytochrome \( c_{550} \), pseudoazurin, or horse heart cytochrome \( c \), in combination with any of the three electron acceptor proteins, nitrite, oxygen, or hydroxylamine \( (3, 13) \). Cytochrome \( cd_1 \) can act as an oxidase; and in the early stages of this reaction an oxidized state of the protein with His/Met coordination at the c heme has been observed \( (10, 15) \), as was also the case for a form of the enzyme seen after a 1-electron oxidation of cytochrome \( cd_1 \) by nitrite \( (5) \). His/Met coordination of the heme of the c domain in the oxidized state is also found in solution for both the semi-apo form of the protein that has lost the \( d_1 \) heme \( (15) \) and the c domain expressed in isolation \( (16) \).

Although the combination of the observations on the catalytic activity of \( P. \) \textit{pantotrophus} cytochrome \( cd_1 \) and the structure of the \( P. \) \textit{aeruginosa} enzyme suggests that bishistidinyl coordination at the c heme and tyrosine ligation at the \( d_1 \) heme may not be significant in steady-state catalysis, there remain many unanswered questions about structure-function relationships for \( P. \) \textit{pantotrophus} cytochrome \( cd_1 \), particularly with respect to the role of tyrosine 25. For example, in a rapid reaction study starting from fully reduced enzyme and nitrite, nitric oxide was not released from the enzyme, suggesting that some conformational change was needed to trigger this event \( (Ref. 5 \) and see also \( Ref. 17) \). Structures of cytochrome \( cd_1 \) from crystals that had first been reduced and then treated with nitrite showed tyrosine 25 apparently poised ready to displace a bound nitric oxide \( (9) \); the significance of this residue, but not necessarily its religation to the \( d_1 \) heme iron, for this displacement has been indicated by calculations \( (17) \). The driving force for tyrosine 25 to ligate to the oxidized iron of the \( d_1 \) heme is certainly large. Cytochrome \( cd_1 \) from \( P. \) \textit{pantotrophus} is exceptional in binding cyanide only when the \( d_1 \) heme iron is in the ferric state; cyanide, which normally binds very tightly to ferric hemes, is readily displaced by the tyrosine residue \( (18) \). Finally, there appears to be a very tight coupling between both the oxidation states and coordination at the c and \( d_1 \) centers that is most clearly illustrated by the highly cooperative and hysteretic redox titration \( (19) \).

It is clear that many aspects of further progress in understanding cytochrome \( cd_1 \) from \( P. \) \textit{pantotrophus} will require the preparation of enzyme carrying mutations at the important residues that have been identified by crystallography. This is no easy task, as the very specialized \( d_1 \) heme is only made by denitrifying bacteria, none of which is an ideal host for site-directed mutation studies. One approach is to make the semi-enzyme \textit{in vivo} and then add the \( d_1 \) heme \textit{in vitro}. This has been done for the enzyme from \( P. \) \textit{aeruginosa}, but it requires the preparation of large amounts of wild-type enzyme as a source of the \( d_1 \) heme \( (e.g. \ Ref. 6) \). Production of an inactive \( cd_1 \) in \( P. \) \textit{pantotrophus} itself is problematic, as recent work on the expression of the \textit{nirS} gene has shown that its transcription is activated by nitric oxide, the product of the reaction catalyzed by the enzyme \( (20) \). Furthermore, anaerobic conditions are needed for formation of cytochrome \( cd_1 \), but use of nitrate as an anaerobic electron acceptor in the absence of an active cytochrome \( cd_1 \) results in accumulation of toxic nitrite.

In view of these considerations, we sought in this work to identify a variant form of \( P. \) \textit{pantotrophus} cytochrome \( cd_1 \) that was still active and that might therefore be expressed in \( P. \)
pantotrophus itself. The recent evidence from several types of study that tyrosine 25 might not be essential for catalysis prompted us to investigate other residues that might be tolerated at this position. Initially, serine was chosen because the P. aeruginosa structure has the hydroxyl of tyrosine 10 at approximately the same distance from the d1 heme iron as we estimated the serine hydroxyl would be in a putative Y25S mutant of the P. pantotrophus enzyme. Thus, we anticipated that an active and stable folded form of the latter enzyme, perhaps with a hydroxide lying between the serine and the heme iron, analogous to the hydroxide that is the immediate ligand to the d1 heme of the P. aeruginosa enzyme, would be produced. This study reports that this expectation of expression of a mutant form of P. pantotrophus cytochrome cd1 was realized and the properties, including the structure, of the Y25S enzyme.

EXPERIMENTAL PROCEDURES

Media, Strains, and Plasmids—Escherichia coli and P. pantotrophus strains are listed in Table I. Luria broth or agar was routinely used for cultivation of these strains, which was carried out at 37 °C. Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; gentamicin (700 units/ml) 20 μg/ml (or 100 μg/ml for E. coli); and rifampicin, 20 μg/ml. Minimal medium for growth of P. pantotrophus strains was prepared as described by Robertson and Kuenen (21); 20 mM succinate and 20 mM KNO3 were added where appropriate. DNA sequencing was performed using Applied Biosystems BigDye terminators as described by the manufacturer. DNA manipulations and transformations were carried out using standard procedures (22). Restriction and modifying enzymes (New England Biolabs Inc.) were used according to the manufacturer's protocols. Southern hybridizations were carried out in 6× SSC (22), 1× Denhardt's solution, and 50% formamide at 42 °C. The nirS gene from P. pantotrophus was mutated such that an EcoRI site was inserted immediately before the start codon, and a HindIII site was inserted immediately after the stop codon (23); the plasmid containing this construct is pEG10 (this has the nirS open reading frame as well as 125 bp upstream DNA (the nirS promoter) and ~600 bp downstream DNA (the start of the nirE gene)). This cassette was then used for further manipulations and mutagenesis of tyrosine 25 to serine. Site-directed mutagenesis was carried out according to Kunkel and Roberts (24) using the mutagenic primer 5’-GTCCAGCGAGGCCTGCAGCGGTGTTGCTGG-3’ with a hydroxide lying between the serine and the heme iron, analogous to the hydroxide that is the immediate ligand to the d1 heme of the P. aeruginosa enzyme, would be produced. This study reports that this expectation of expression of a mutant form of P. pantotrophus cytochrome cd1 was realized and the properties, including the structure, of the Y25S enzyme.

| Strain | Characteristics | Source/Ref. |
|--------|-----------------|-------------|
| LMD92.63 | Spontaneous rifampicin-resistant strain | Laboratory stock |
| EFPf1r | PstI site was end-filled | This study |
| EG2602 | XbaI site was end-filled | This study |
| DH5a | supE44 lacU169 (d800 lacZ Δ153) hadR17 recA1 endA1 gyrA96 thi-1 relA1 | Invitrogen |
| S17-1 | thi pro havR havM’ recA, chromosomal insertion of RP4-2 (Tc::Mu Km::Tn7) | 38 |
| pJQ200KS | Gene replacement plasmid | This study |
| pTZ19r | Cloning vector | This study |
| pEG10 | pTZ19r, carrying 2.5-kb PstI-SphI genomic clone of nirS, internal EcoRI and HindIII sites removed, and EcoRI and HindIII sites added before and after start and stop codons, respectively | This study |
| pEG156 | pEGEM52T PstI-SphI fragment from pEG10 | This study |
| pEG181 | pJQ200KS nirS:aph | This study |
| pEG211 | Expression vector, tac promoter, Km’ | This study |
| pEG276 | Expression vector, rnh promoter, Gm’ | This study |
| pEG607 | pTZ19r nirS (Y25S) | This study |
| pEG760 | pEG276 nirS (Y25S) | This study |

Construction of Plasmids for Production of NirS Derivatives—A custom expression vector was constructed to facilitate expression of nirS and its derivatives in P. pantotrophus. The backbone of this vector was the 5-kb Csp45l-AatII restriction fragment from the broad host range vector pBBR1MCS (27). The gene encoding kanamycin resistance and fragments coding for the lacI gene, tac promoter, and rnb terminators were amplified using PCR methodology from plasmids pHRP310 (28) and pMMB300EH (29), respectively. Restriction enzyme sites were added to the oligonucleotide primers such that the amplified products had the following constructions: Csp45l-lacI-Nhel, XbaI-aph-SacII, and SacII-Pm-Rrrl-AatII. Ligation of these fragments with the 5-kb Csp45l-AatII restriction fragment from pBBR1MCS generated plasmid pEG211, which thus carried the inducible tac promoter, with the lacI gene immediately downstream of the kanamycin gene, such that transcription from the aph gene also resulted in transcription of the lacI gene. A derivative of this plasmid, pEG276, consisted of the aac gene (coding for resistance to gentamycin) from pHRP309 (28) instead of the aph gene. A NsiI restriction site was also inserted immediately before the multiple cloning site, enabling the promoter driving overexpression (in this case, the rnh promoter from the rnah operon of Rhodobacter sphaeroides) (30) to be changed easily. This promoter was obtained using primers rnhF and rnhR (5’-CAACCGCCGGCTCCCGGACGCGGCTGGT-3’) and rnhR (5’-ACAATGACGTCGACCCGTGAGT-3’) such that the former incorporated a SacII restriction site and the latter a NsiI restriction site. Thus, the amplified fragment could be transferred into a promoter-free expression vector to give pEG276.

Growth of Cells and Purification of the Y25S Protein—The inoculum for the large-scale culture (as described above) was obtained by first inoculating 50 ml of Luria broth culture (in a 250-ml flask) with a colony from a Luria broth plate that had been freshly streaked with a glycerol stock 2 days earlier. This 50-ml culture was grown for ~15 h at 37 °C with shaking; 5–10 ml was then used to inoculate a 5-liter culture for enzyme preparation.

For the purification of Y25S cytochrome cd1, P. pantotrophus cells carrying plasmid pEG607 were grown overnight (~17 h) at 37 °C in minimal medium with 20 mM succinate and 20 mM KNO3 under the selective pressure of 20 μg/ml gentamicin and 50 μg/ml kanamycin. It was crucial that diffusion of atmospheric oxygen into the culture was minimized. Hence, either fully filled 5-liter Erlenmeyer flasks with narrow necks and thus small surface area or 5-liter Duran bottles filled up to the neck with minimal surface area were used to ensure that the culture was anaerobic as possible. If a larger surface area of the culture was exposed, the then increased rate of oxygen transfer into the respiring culture resulted in formation of mainly semi-apoenzyme lacking the d1 heme.

Harvesting and purification procedures were similar to those used for the wild-type enzyme (31), i.e., a periplasmic preparation was first made using lysozyme. The periplasmic proteins were then loaded onto a DEAR-Sepharose fast flow column. Fractions (eluted by a gradient of 0.1–0.5 M NaCl) containing cytochrome cd1, as judged by visible absorb-
Data collection

Space group: P2₁

Cell dimensions:
- a = 106.797 Å
- b = 60.812 Å
- c = 100.340 Å
- γ = 112.33°

Resolution range (Å):
- 30–1.4 (1.45–1.40)

No. of unique reflections: 812,906

No. of reflections:
- I = 233,907
- I > 2σ(I) = 24.8 (4.72)

Rmerge (%): 4.8 (24.7)

Completeness (%): 100 (100)

Statistics for final model

- No. of amino acid residues: 1118 (chains A and B)
- No. of solvent molecules: 1370 (1355 water molecules, 8 sulfate ions, 7 glycerol molecules)
- R-factor (%): 15.9
- Free R-factor (%): 17.5
- Average B-factor: 10.9
- Protein atoms (Å²): 12.7
- All atoms (Å²): 12.7
- r.m.s.d.: 0.012
- For bond lengths (Å): 1.4°
- For bond angles: 1.4°

Numbers in parentheses refer to the outer shell in data collection.

Results

Expression of the Protein—Cells of P. pantotrophus ΔnirS carrying the Y25S mutant protein grew under conditions similar to those used for the wild-type enzyme (31), i.e., from a solution containing 10–20 mg/ml protein in the presence of 2.2–2.4 m ammonium sulfate and 50 mM potassium phosphate (pH 7.0). Data to a resolution of 1.4 Å were collected at 100 K using monochromatic x-rays at a wavelength of 0.934 Å on beam line I711 at the MAX Synchrotron (Lund, Sweden). The refined model of the oxidized protein was used for initial phasing (7). The phases were refined using the programs REFMAC (32) and CNS (33), and model building was done with the program O (34). The overall crystallographic R-factor for the final model was 15.9%, and the free R-factor was 17.5% (Table II).

Other Methods—Electrospray ionization mass spectrometry was performed on a Micromass Bio-Q II-2S triple quadrupole atmospheric pressure instrument equipped with an electrospray interface. Samples were introduced via a loop injector as a solution (20 pmol/µl in 1:1 water acetonitrile and 1% formic acid) at a flow rate of 10 µl/min. Visible absorption spectra were obtained using a PerkinElmer Life Sciences 12 spectrophotometer. Steady-state kinetic assays were performed at 25 °C and pH 7.0 in 50 mM potassium phosphate buffer as described previously (3) using cuprous pseudoazurin as electron donor.

Expression of the Protein—Cells of P. pantotrophus ΔnirS carrying the Y25S variant of cytochrome cd₁ grew under anaerobic conditions at a similar initial rate, but to a lower final cell density, as those carrying a plasmid coding for the wild-type enzyme. However, although growth of both wild-type cells and ΔnirS cells carrying the Y25S mutation required anaerobic conditions for production of cytochrome cd₁, only the latter required extra precautions to minimize entry of oxygen into the cultures (see “Experimental Procedures”). The anaerobic cultures of cells containing the Y25S variant of cytochrome cd₁ evolved copious amounts of gas, presumably nitrogen. This observation is indicative of denitrification proceeding past the reduction of nitrite, which accumulated transiently, to nitric oxide. Cells carrying the deletion in the chromosomal copy of nirS, but without a plasmid coding for an active enzyme, grew, without gassing, to only restricted cell densities, and nitrite had accumulated in the medium at the end of growth. Thus, it can be deduced that the Y25S variant enzyme is sufficiently active in vivo to sustain growth of cells under conditions that require reduction of nitrite.

Electrospray Mass Spectrometry—The calculated mass (including the covalently bound heme, but not the noncovalently bound d₁ heme) of the Y25S variant of P. pantotrophus cytochrome cd₁ is 63,022 Da. Electrospray mass spectrometry of the Y25S protein gave a value of 63,009 ± 1.5 Da. The interpretation of the difference between this value and the calculated value is that the N-terminal glutamine has cyclized with the elimination of a molecule of ammonia (17 Da). This conclusion was confirmed by failure to obtain an N-terminal amino acid sequence by Edman degradation. Such cyclization has been observed previously for wild-type P. pantotrophus cyto-

Table II

| Data collection | P₂₁ |
|-----------------|-----|
| a = 106.797 Å | 60.812 Å | 100.340 Å | 112.33° |
| Resolution range (Å) | 30–1.4 (1.45–1.40) |
| No. of unique reflections | 812,906 |
| No. of reflections | 233,907 |
| σ(I) | 24.8 (4.72) |
| Rmerge (%) | 4.8 (24.7) |
| Completeness (%) | 100 (100) |

Statistics for final model

- No. of amino acid residues: 1118 (chains A and B)
- No. of solvent molecules: 1370 (1355 water molecules, 8 sulfate ions, 7 glycerol molecules)
- R-factor (%): 15.9
- Free R-factor (%): 17.5
- Average B-factor: 10.9
- Protein atoms (Å²): 12.7
- All atoms (Å²): 12.7
- r.m.s.d.: 0.012
- For bond lengths (Å): 1.4°
- For bond angles: 1.4°

Numbers in parentheses refer to the outer shell in data collection.

FIG. 2. Structure of P. pantotrophus cytochrome cd₁, in its oxidized (as prepared) form (7). The figure is oriented such that the mainly α-helical c-type cytochrome-binding domain is at the top, with the eight-bladed β-propeller d₁, heme-binding domain below; the hemes are shown in black. In the oxidized enzyme structure shown here, the c heme ion has His₁⁷ and His₁⁸ as axial ligands; the d₁ heme is ligated by His₁²⁰⁰ and Tyr₁³⁸. The overall structure of the Y25S variant is essentially identical to that of the wild-type enzyme, with small differences only in the vicinity of the mutated residue (see “Results”); His/His coordination of the c heme is retained, whereas the d₁ heme is axially ligated by His₁²⁰⁰ and a sulfate ion.
Confirmation of this cyclization cannot be obtained from the crystal structure of the *P. pantotrophus* protein (wild-type or Y25S variant) because the first eight residues at the N terminus are disordered (Ref. 6 and see below). The 1.4-Å crystal structure of the Y25S variant (see below) clearly shows that there are no other amino acid changes throughout the protein, and the DNA sequence of the disordered part of the N-terminal arm region also indicates that it does not contain alterations in that region. Thus, the difference between the expected molecular mass (63,022 Da) and the determined value (63,009 Da) can be attributed with some confidence to the cyclization of the N-terminal residue.

**Structure of the Y25S Mutant**—The structure of the Y25S mutant of oxidized *P. pantotrophus* cytochrome cd₁ was determined to a resolution of 1.4 Å. The overall structure is essentially identical to that of the wild-type oxidized enzyme (Fig. 2).
The latter. The presence of sulfate bound to the utes to stabilizing the predominant position of the side chain of sulfur atom of methionine 409 is near to serine 25 and contrib-
tions of the serine side chain. It is notable that the interactions are included. The buried surface area was calculated using a probe radius of 1.4 Å.

Interactions between the N-terminal arm (residues 9–48) of the enzyme up to residue 135 (the N-terminal arm and the cytochrome c domain) and the d1 (C-terminal) domain; only direct electrostatic interactions are included. The buried surface area was calculated using a probe radius of 1.4 Å when all Cα atoms are superimposed. There were additional differences in the proximity of the mutated residue. The electron density shows unambiguously that residue 25 has been mutated to serine. In both subunits, the serine 25 side chain adopts two conformations, which have approximate fractional occupancies of 0.7 and 0.3 respectively (Fig. 3, A and B). Unlike for tyrosine 25 in the wild-type enzyme, the hydroxy group of serine 25 in the Y25S variant is not directly bonded to the iron atom of the d1 heme. Electron density extending from the d1 heme iron was interpreted as a sulfate ion (Fig. 3, c).

The solution spectrum of the oxidized Y25S variant, as observed for wild-type P. aeruginosa cytochrome c552 (10), and a sulfate ion heme ligand was also reported in the structure of oxidized pentaheme (NfA) nitrite reductase (35).

The consequence for the structure of replacement of tyrosine 25 by serine was remarkably localized to the immediate vicinity of the distal side of the d1 heme. There was no effect on the position of the proximal ligand, histidine 200, or on the positions of residues 24 and 26 (Fig. 3C). Indeed, surprisingly, the conformation of the entire structurally ordered part of the N-terminal arm (residues 9–48) (7) was unaffected by the mutation. All noncovalent interactions, other than those associated with Tyr25, between the N-terminal arm and both the c and d1 domains are present in the mutant structure. In particular, the unusual bishistidinyl coordination of the c-type cytochrome center of the oxidized state of the wild-type enzyme, with the two imidazole groups almost perpendicular to each other, is retained in the Y25S mutant (Fig. 4).

Visible Absorption Spectra—The solution spectrum of the oxidized Y25S protein differed dramatically from that of the wild-type protein at the wavelengths characteristic of the d1 heme. There was no band comparable to the high-spin d1 heme absorption at 702 nm that is found for the wild-type enzyme, but rather a broad absorbance with its maximum at ~640 nm (Fig. 5a). This indicates that the d1 heme is all low-spin in the Y25S variant, as observed for wild-type P. aeruginosa and Pseudomonas stutzeri cytochromes cd1 (12). Note that for wild-type P. pantotrophus cytochrome cd1, the His/Tyr-coordinated d1 heme is in a high-spin/low-spin thermal equilibrium around...

| Structure No. of interactions Buried surface area PDB code |
|-----------------|-----------------|----------------|
| **Oxidized P21** | **Subunit A** | 19 (10)² | 2740² | 1QKS |
| **Subunit B** | 20 (11)² | 2790² | |
| **Reduced P21** | **Subunit A** | 3 (0) | 650 | 1AOE |
| **Subunit B** | 13 (7)³ | 1430 | |
| **Reduced P1,2,2** | **Subunit A** | 2 (0) | 1340 | 1H9X |
| **Subunit B** | 3 (0) | 1370 | |

² Replacement of Tyr25 with Ser results in loss of one interaction in each of subunits A and B.

Fig. 4. Bishistidinyl coordination of the c heme in the oxidized state of the Y25S variant of cytochrome cd1 from P. pantotrophus.
The wild-type enzyme (3). The both the wild-type and Y25S mutant enzymes when both were pseudoazurin as electron donor and nitrite, oxygen, or hydroxylamine were so similar (Fig. 5 spectra of the reduced wild-type and Y25S variant enzymes kinematic constants for the Y25S enzyme.

\[ \text{Extinction coefficient for reduced wild-type protein} = 323 \text{ mM cm}^{-1} \text{ at 418 nm} \]

Absorption by the \( d_1 \) heme in the reduced Y25S enzyme (see b) was ~10% less than that in the wild-type enzyme. Because the shapes of the two spectra are otherwise identical, we attribute this to the greater tendency of the Y25S variant to lose the \( d_1 \) heme (see also “Discussion”). The buffer used was 50 mM potassium phosphate (pH 7.0), and the temperature was 25 °C.

In contrast, the absorption spectrum of the reduced state of the Y25S variant protein was very similar to that of reduced wild-type P. pantotrophus cytochrome \( cd_1 \), as illustrated in Fig. 5b. The characteristic (15) split a-band at ~550 nm, with the higher intensity at the shorter of the two wavelengths, was retained. These data suggest that the reduced states of the two forms of cytochrome \( cd_1 \) are similar in terms of the coordination and environment of both types of heme center in the enzyme.

**Steady-state Kinetic Assays—**Assay with P. pantotrophus pseudoazurin as electron donor and nitrite, oxygen, or hydroxylamine as acceptor showed that the activities were similar for both the wild-type and Y25S mutant enzymes when both were pretreated by reduction with dithionite, which activates the wild-type enzyme (3). The \( k_{\text{cat}} \) values (expressed per monomer of cytochrome \( cd_1 \)) for nitrite, oxygen, and hydroxylamine were 67, 6.2, and 7.7/s for the Y25S mutant and 68, 3.2, and 3.2/s for the wild-type enzyme, respectively. However, if a pre-reduction step was omitted, then whereas the \( k_{\text{cat}} \) values for the wild-type enzyme were reduced by 96% (3), there was no attenuation of the activity of the mutant enzyme. The \( K_m \) for nitrite was 62 \( \mu \)M for the Y25S protein, a similar value to that (71 \( \mu \)M) observed for the activated wild-type enzyme (3). Because the absorption spectra of the reduced wild-type and Y25S variant enzymes were so similar (Fig. 5b), the extinction coefficient for reduced wild-type P. pantotrophus cytochrome \( cd_1 \) (323 mM cm\(^{-1} \) at 418 nm) (15) was used to calculate concentrations of (and hence, kinetic constants for) the Y25S enzyme.

**DISCUSSION**

**Significance of Tyrosine 25 Binding to the \( d_1 \) Heme Iron—**When bishistidinyl coordination of the c-type cytochrome center and tyrosine 25 as a ligand to the \( d_1 \) heme iron were first observed in crystals of oxidized cytochrome \( cd_1 \) from P. pantotrophus, it was thought that the tyrosine residue may play a key role in displacing the reaction product, nitric oxide, from the \( d_1 \) heme active site (7). Two structures of the reduced enzyme show that the tyrosine is displaced from the active site, consistent with opening up the \( d_1 \) heme iron for binding of nitrite, and that the heme iron coordination at the c center has switched to histidine/methionine (10, 11). These other studies (13, 15), along with the unusual hysteretic redox titration that demonstrated considerable cooperativity between the c and \( d_1 \) centers (18), have all suggested that ligation of tyrosine 25 to the \( d_1 \) heme is strongly coupled to the bishistidinyl coordination at the c heme center.

The properties of the Y25S mutant protein that are reported here show that the coordination of tyrosine 25 to the \( d_1 \) heme is not required per se to trigger the adoption of His/His coordination at the c heme center. Even without Tyr25, there are sufficient other residues to make contacts (see below) to maintain the N-terminal arm structure that is characteristic of the crystalline state of the oxidized (as prepared) protein. This consideration explains why one crystal form seen previously with nitric oxide, rather than tyrosine 25, bound at the \( d_1 \) heme could still have His/His coordination at the c-type center (9). In the latter structure, tyrosine 25 could be seen at the edge of the \( d_1 \) heme-binding site.

The finding that, with a physiological electron donor as substrate, the Y25S protein is as active a nitrite reductase as the wild-type protein does not support the earlier proposal that Tyr25 plays a role in driving the dissociation of each nitric oxide from the active site of the enzyme through a steady-state cycle of direct coordination on to and release from the iron of the \( d_1 \) heme (7). However, it is still possible, for example, that approach of tyrosine 25 toward the two active-site histidine residues (positions 345 and 388) plays a role in driving dissociation of nitric oxide (17) and that this role can be replicated by the hydroxy group of serine 25. In other words, as discussed elsewhere (17), the return of Tyr25 to a position between His345 and His388 may facilitate product (nitric oxide) release, but recombination of Tyr25 to the oxidized iron of the \( d_1 \) heme is bypassed during steady-state catalysis. As cytochromes \( cd_1 \) from various sources have different ligands that can approach the \( d_1 \) heme iron, a range of ligands (e.g., Tyr25 or Ser25) may interact with the conserved His345 and His388 so as to create an unfavorable orientation for nitric oxide and thus promote its dissociation (17).

The lack of requirement for a reductive pre-activation of the Y25S enzyme, in marked contrast to the wild-type enzyme (3), is intriguing. It is probable that the reduction potential of the bishistidinyl-coordinated heme is less positive than that of the electron donor proteins pseudoazurin and cytochrome c550 (19). However, this need not prevent electron transfer from these donors via the c domain heme center and on to the \( d_1 \) heme active site if the overall reaction is exergonic. As discussed by Page et al. (36), there are many examples of uphill energetic steps in chains of electron carriers. Tyrosine is known to stabilize the ferric form of heme groups; thus, the absence in the Y25S mutant of the phenolic oxygen ligand to the \( d_1 \) heme provided by tyrosine may raise the reduction potential of the \( d_1 \) heme sufficiently to make it reducible by electrons originating from pseudoazurin. Hence, the triggering of dissociation of tyrosine 25 by reduction with dithionite that is needed to activate the wild-type enzyme (3) would not be required for the Y25S mutant. Alternatively (or additionally), the adoption of His/Met coordination of the c heme by the wild-type enzyme may be dictated, at least in part, by the requirement for con-
comitant removal of tyrosine 25 from its position blocking the entry of nitrite into the d1 heme active site (3). In the Y25S mutant, the serine is less effective at blocking the active site because it does not directly coordinate the d1 heme iron. Nitrite binding to the d1 heme iron and its reduction to nitric oxide (E° = +374 mV) provide a final step in the electron transport chain that is thermodynamically favored relative to the reduction potential of the electron donor proteins cytochrome c550 and pseudoazurin, for which E° is approximately +250 mV. It is clear that definitive answers to these issues will require substantial further work, which may also reveal whether P. pantotrophus cytochrome cd1 differs from or is similar to the counterpart in P. aeruginosa, where reduction of the d1 heme before the c heme, at least in the crystal, is linked to the conformational change that drives hydroxide from the active site (37).

The N-terminal Arm is an Important Structural Element in the Oxidized Monoclinic Structure—A comparison of domain-domain interactions in the known conformers of P. pantotrophus cytochrome cd1 shows that the structure of the oxidized enzyme derived from monoclinic (P2 1) crystals has the largest domain-domain interface (Table III). The interface is stabilized by 19 or 20 hydrogen bonds and salt bridges between the N-terminal part of the enzyme and the d1 domain in subunits A and B, respectively. Of the 19 (20 in subunit B) interactions between the N-terminal part and the d1 domain, 10 (11 in subunit B) are mediated by the N-terminal arm (residues 9–48). Moreover, the N-terminal arm provides heme ligands to both the d1 and c hemes, and it thus constitutes an important structural element in the oxidized monoclinic structure. As can be seen in Table III, the replacement of Tyr25 by serine causes loss of only one interaction. It is tempting to conclude from this observation that the binding of Tyr25 to the d1 heme iron is not critical for the conformation of the N-terminal arm that permits bishistidinyl coordination of the c heme. Rather, it appears possible that, in P. pantotrophus cytochrome cd1, it is reduction of the iron of the c heme that triggers the movement of this arm and the recruitment of Met106 as a ligand to the iron of this center. This would be consistent with the observation (e.g. Ref. 19) that, in general, oxidized (Fe(III)) heme is thermodynamically stabilized relative to Fe(II) heme by His/His coordination, whereas Fe(II) heme is relatively stabilized by His/Met coordination. However, Table III also shows that, in the more recently described tetragonal crystal form of P. pantotrophus cytochrome cd1 (10), there are far fewer contacts between the N- and C-terminal domains, in either the oxidized or reduced state. At present, the relative contributions to steady-state catalytic turnover of solution structures similar to those seen in the different crystal forms cannot be evaluated.

If neither tyrosine 25 ligation to the d1 heme nor bishistidinyl coordination of the c heme plays a role in the catalytic activity of cytochrome cd1 from P. pantotrophus (3, 13–15), we are left with the task of seeking an alternative explanation for the occurrence of these structural features. As noted above, they are related to a significant stabilization of the N-terminal domain in the oxidized crystal and can also be deduced to occur in solution (12) on the assumption that their presence correlates with bishistidinyl coordination at the c heme center. Furthermore, the oxidized wild-type enzyme seems unable to bind cyanide in solution; in common with the crystal, only the reduced state of the enzyme binds this ligand in solution (18). Thus, the strong driving force for adoption of the His/His-coordinated c heme by the oxidized enzyme is not restricted to the crystalline state. On the other hand, the recent demonstration, by a combination of solution spectroscopies (13, 14), that a His/Met-coordinated c heme conformer of the oxidized enzyme can persist in solution for many minutes after the addition of hydroxylamine to the reduced enzyme might suggest that the bishistidinyl-coordinated c-type center and the tyrosine-ligated d1 heme center are less favored, at least in a kinetic sense, in solution than in the crystal. Cells carrying the Y25S mutation do not grow to the same density as those carrying the same plasmid with the wild-type gene inserted. We have also found that it is crucial to minimize the diffusion of atmospheric oxygen into the culture producing the Y25S variant protein (see “Experimental Procedures”). This finding is consistent with an earlier suggestion (10) that, when a depletion of reducing equivalents occurs in the periplasm, dioxygen or peroxides may react with an open/unprotected d1 heme, creating harmful side reactions. Shutting off access to the d1 heme by a return of Tyr25 to the iron would prevent the heme iron from reacting with oxygen species in an uncontrolled manner. Such a “shutoff” mechanism is not possible in the Y25S protein. We found that an enhanced rate of oxygen transfer into the respiring culture resulted in formation of mainly semi-apo-enzymes, which lacks the d1 heme. The Y25S enzyme obtained immediately after cell breakage is contaminated to a greater extent than the wild-type protein by semi-apatrotein. It therefore seems likely that the presence of Tyr25 is advantageous because it stabilizes the protein, particularly with respect to the initial insertion and/or subsequent retention of the d1 heme.

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