Does the Reduction of c Heme Trigger the Conformational Change of Crystalline Nitrite Reductase?*

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The structures of nitrite reductase from Paracoccus denitrificans GB17 (NiR-Pd) and Pseudomonas aeruginosa (NiR-Pa) have been described for the oxidized and reduced state (Fülöp, V., Moir, J. W. B., Ferguson, S. J., and Hajdu, J. (1995) Cell 81, 369–377; Nurizzo, D., Silvestrini, M. C., Mathieu, M., Cutruzzolà, F., Bourgeois, D., Fülöp, V., Hajdu, J., Brunori, M., Tegoni, M., and Cambillau, C. (1997) Structure 5, 1157–1171; Nurizzo, D., Cutruzzolà, F., Aresè, M., Bourgeois, D., Brunori, M., Cambillau, C., and Tegoni, M. (1998) Biochemistry 37, 13987–13996). Major conformational rearrangements are observed in the extreme states although they are more substantial in NiR-Pd. The four structures differ significantly in the c heme domains. Upon reduction, a His87/Met106 heme-ligand switch is observed in NiR-Pd together with concerted movements of the Tyr in the distal site of the d1 heme (Tyr10 in NiR-Pa, Tyr25 in NiR-Pd) and of a loop of the c heme domain (56–62 in NiR-Pa, 99–116 in NiR-Pd). Whether the reduction of the c heme, which undergoes the major rearrangements, is the trigger of these movements is the question addressed by our study. This conformational reorganization is not observed in the partially reduced species, in which the c heme is partially or largely (15–90%) reduced but the d1 heme is still oxidized. These results suggest that the d1 heme reduction is likely to be responsible of the movements. We speculate about the mechanistic explanation as to why the opening of the d1 heme distal pocket only occurs upon electron transfer to the d1 heme itself, to allow binding of the physiological substrate NO2– exclusively to the reduced metal center.

The biological reactions involved in denitrification are not yet completely understood at the molecular level. Bacteria, like Pseudomonas, Paracoccus, or Desulfovibrio, can grow on nitrate as sole nitrogen source and can use nitrate as terminal electron acceptor under anaerobic growth conditions (5). Four elementary steps take place for the reduction of nitrate to N2, catalyzed by nitrate, nitrite, nitric oxide, and nitrous oxide reductase, respectively (6). Special attention has been devoted to nitrite reductases (NiR) because these enzymes catalyze in vitro the four-electron reduction of dioxygen with formation of H2O, as typical of membrane-bound terminal oxidases, and in vivo catalyze the electron transfer from cytochrome c553 to nitrite with production of NO. NiR-Pa is purified from the periplasmic space as an homodimer of 120 kDa, carrying one c heme and one d1 heme per subunit. The c heme is reduced first and the d1 heme is the site of reduction of oxygen (7, 8) and nitrite (9, 10).

We have solved the structure of oxidized NiR-Pa (3). The d1 heme domain is an eight-bladed β-propeller in which a β-sheet of four antiparallel β-strands forms each blade, and the c heme domain has a typical class I cytochrome c fold, with a His51-Met88 iron coordination. A peculiar feature in NiR-Pa is the “domain swapping” (11, 12) of the N-terminal region (6–29), which brings Tyr10 of the one monomer close to the d1 heme site of the other. Tyr10 is hydrogen-bonded by the OH side chain to a hydroxide ion that is the sixth ligand of the d1 heme iron and hinders the access of the catalytic site to the substrate. In NiR from Paracoccus denitrificans GB17 (NiR-Pd) on the other hand, the c heme has an unusual His17-His88 coordination in the oxidized state; moreover, the “domain swapping” is absent and Tyr25 of the c domain of the same monomer directly coordinates the d1 heme iron (1).

Very unusual in the field of redox proteins, the NiR conformation largely depends on the redox state of the protein, and major changes in the structural organization were found to occur upon reduction. The structure of reduced NiR-Pd recently published (2) shows large rearrangements at the level of the c domain: the N-terminal arm from 26 (or 36) back is disordered after reduction, and in particular Tyr25, a ligand of the d1 heme Fe(III) is pushed out of the site upon reduction. Moreover, His17, one of the two c heme ligands, is replaced by Met106 brought in by the significant movement (15–17 Å) of the loop (99–116). In the structure of the reduced NiR-Pa (NiR-red) (4), the rearrangements are of smaller amplitude and consist of coordinated movements (Fig. 1): (i) a 6 Å rocking movement of the loop (56–62) toward the c heme, likely stabilized by the formation of new hydrogen bonds between Thr59 and Gln11 and between Ala58 and Gly60, in the middle of this loop, which tightens the loop in the reduced form; (ii) the rotation of Tyr10 (4.2 Å) and the disappearance of the hydroxide ion coordinating the d1 heme iron, which make the d1 heme site accessible to the substrate. These movements are independent of the presence of NO at the active site (4). Because an identical movement of

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The atomic coordinates and structure factors (codes 1N15, 1N50, and 1N90) have been deposited in the Protein Data Bank, Brookhaven National Laboratory, Upton, NY.

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¶The abbreviations used are: NiR, nitrite reductase; NiR-Pa, nitrite reductase from Pseudomonas aeruginosa; NiR-Pd, nitrite reductase from Paracoccus denitrificans; NiR-red, reduced NiR; NiR-ox, oxidized NiR; r.m.s., root mean square.
Reduction of c Heme Domain of Nitrite Reductase

Tyr\textsuperscript{10} was also present when the d\textsubscript{1} heme was incompletely reduced, we proposed (4) that the loop (56–62) displacement occurs upon reduction of the c heme and triggers a cascade of movements.

In the present paper, we directly address the question of whether these conformational changes are visible in an enzyme having the d\textsubscript{1} heme oxidized while the c heme is already reduced. With this aim, we have investigated the x-ray structure of NiR-Pa at different discrete levels of c heme reduction, all having the d\textsubscript{1} heme oxidized. This experiment has taken advantage of the slow intramolecular electron transfer from c heme to d\textsubscript{1} heme in NiR-Pa, which has been already characterized in solution and confirmed here in the crystalline state. The partially reduced forms of NiR-Pa were obtained by soaking the oxidized crystals in the presence of ascorbate as reducing agent and by cryoquenching the reaction at different times. The redox state of the enzyme was assessed by microspectrophotometry and the potential structural rearrangements being analyzed by the x-ray method.

EXPERIMENTAL PROCEDURES

Crystallization and Soaking Procedure—The protein used for the crystallization was purified following the method by Parr (13). It had the expected absorbance ratio in the oxidized state (A\textsubscript{541 nm}/A\textsubscript{280 nm} ≥ 1.0, A\textsubscript{411 nm}/A\textsubscript{280 nm} ≥ 1.2) and showed a single SDS-polyacrylamide gel electrophoresis band. The crystallization conditions were described previously (3). Orthorhombic crystals (P\textsubscript{2}\textsubscript{1}2\textsubscript{1}2\textsubscript{1}) were obtained in 2 M Na/K\textsubscript{2}-phosphate, 50 mM Tris-HCl, pH 8.4, and had cell parameters of (163.1 × 90.1 × 111.9) Å \textsuperscript{3} (3). Oxidized crystals were soaked in a solution containing 25 or 50 mM Na-ascorbate in 2 mM Na/K\textsubscript{2}-phosphate and 50 mM Tris-HCl, pH 7.2. Crystals NiR-15 and NiR-50 were soaked in 25 mM Na-ascorbate for 6 and 30 min, respectively; whereas crystal NiR-90 was soaked in 50 mM Na-ascorbate for 7 min. After soaking, the crystals were transferred for a few seconds to solutions containing 15% glycerol. All the solutions were previously saturated with argon.

Microspectrophotometry—In the microspectrophotometric experiments, care was taken to record optical densities in the range of linearity (OD ≤ 2.5). The ORIEL microspectrophotometer mounted on the ID09 beamline at the ESRF consisted of a xenon lamp source and a CCD detector. The spectra were recorded with unpolarized light. The dark current corresponding to the electric noise was measured, and the base-line correction was performed with a buffer similar to that in which the crystals were soaked. The crystals were taken from the soaking solution, mounted on a cryoloop, and frozen in a cold stream of nitrogen gas. The cryoloop put on the goniometer head was maintained at 100 K by a nitrogen cryostream (Oxford Cryosystems). Spectral processing (smoothing, displaying, and page setting) was performed with the IDL5.1 (Research System, Inc.) and Xmgr3.01 (ACE/GR Development Team) software. The spectra of the frozen crystals were normalized at 541 nm, the isosbestic point in the transition between the oxidized and the reduced form. On the normalized spectra, we calculated the percentage of reduction of the c heme as the ratio of ΔOD to the maximum ΔOD observed between the totally oxidized and the totally reduced form at 545 and 551 nm in the α band of the c heme, where the spectral contribution of the d\textsubscript{1} heme is negligible compared with that of the c heme. Three crystals (NiR-15, NiR-50, and NiR-90) at 15, 50, and 90% reduction of the c heme, respectively, were selected for x-ray data collection. A control spectrum was recorded after the exposure of the intermediate crystal at 50% c heme reduction. This spectrum was not significantly different from that taken before exposure to x-rays.

Data Collection, Processing, and Structure Refinement—X-ray data of NiR-15, NiR-50, and NiR-90 were collected at the ID14 beamline at the ESRF (Grenoble, France) on a MarCCD detector, using a 0.933-Å wavelength. A crystal to detector distance of 165 mm was chosen to avoid spot overlap as much as possible. Data sets were indexed and integrated with DENZO (14) and PrOW (15). They were merged with the CCP4 suite (16) at a maximum resolution of 2.70 Å.

The NiR-ox model at 2.15-Å resolution (3) without any ions or water molecules was used as the starting model. To accurately compare with the NiR-red model, which had been refined between 12.0 and 2.90 Å (4), the refinement of NiR-15, NiR-50, and NiR-90 was performed using the same resolution range between 12.0 and 2.90 Å. Moreover, the same reflection set was used for the calculation of the R\textsubscript{free} and an identical refinement procedure was performed on all models and data sets (NiR-15, NiR-50, NiR-90, and NiR-red). After several cycles of the rigid-body refinement procedure in X-PLOER3.8 (17, 18), the two domains of each

FIG. 1. Stereo view of the conformational changes associated with the change of redox state of NiR-Pa. The close environment of the c and d\textsubscript{1} heme in the oxidized (thin line) and the reduced form (thick line) are superimposed.
monomer were satisfactorily fitted. At this stage, we calculated the first 
sigmaA weighted maps (2mFo-DFc, difference Fourier with reduced 
model bias) and (mFo-DFc) (19) (Figs. 3, A–C and 4A). The refinement, 
including some conjugate gradient and B-factors refinement cycles in 
X-PLOR3.8, was alternated with visual inspection on the display, per-
mitting the final model to be obtained. The refinement and stereochem-
ic data are given in Table I, in which the statistics show the good 
quality of the models despite the absence of water molecules. The 
coordinates have been deposited at the Protein Data Bank with codes 
1N15, 1N50, and 1N90, respectively for the NiR-15, NiR-50, and NiR-90 
models and data sets. The r.m.s. deviations were calculated with the 
LSQMAN program (20). Visual inspection and manual rebuilding of the 
model were carried out with the graphic program TURBO-FRODO (21).

**RESULTS**

**Microspectrophotometric Characterization of the Reduction of NiR-Pa Crystals by Ascorbate**—The spectral characterization of NiR-ox and NiR-red in the crystal has been described previously (4). After the addition of ascorbate to the mother liquor bathing the crystal of NiR-ox, spectra between 700 and 400 nm were recorded. Upon reduction, the Soret band maximum shifted from 411 to 417 nm (not shown) and the α and β bands of the c heme become resolved; the maximum of the d1 
heme α band shifted from 640 to 650 nm. The crystals spectra, 
well resolved at low temperature, show the α band of the c 
heme split in two peaks at 545 and 551 nm and the β band 
centered at 519 nm and flanked by two shoulders at 510 and 
525 nm (Fig. 2).

When the crystals of oxidized NiR-Pa are exposed to Na-
ascorbate 50 mM, the reduction of the c heme is fast and almost 
complete in a few minutes, whereas the α band of the d1 heme 
starts shifting toward 650 nm only after several minutes (~10’ 
at room temperature).

On the basis of the reduction time course observed in the 
crystal, it seemed feasible to obtain crystals in which the d1 
heme is oxidized and the c heme is at intermediate levels of 
reduction. We have then selected discrete times at which the 
reduction was stopped by freeze-quenching the crystals. The 
redox state of NiR-Pa in the frozen crystals was checked by 
microspectrophotometry before the x-rays collection (Fig. 2); in 
particular, after having recorded and normalized the spectra at 
541 nm, we calculated the approximate proportion of the c 
heme in the reduced state. The spectra in Fig. 2 correspond to

![Fig. 2. Optical microspectrophotometer spectra of cryocooled crystals (100 K) between 480 and 700 nm. NiR-ox and NiR-red (thick line), forms at approximately 15, 50, and 90% reduced c heme (thin line) and control of the 50% after exposure (dotted line), respectively. The spectra were recorded with unpolarized light. The crystal spectra have been normalized at 541 nm, the isosbestic point in the α-red transition.](image-url)
crystals in which the c heme is approximately at 15, 50, and 90% reduced. In all these spectra, within experimental error limits, the a band of the d1 heme is superimposable to that of the oxidized enzyme, showing that the d1 heme remains oxidized throughout the reduction of the c heme.

The X-ray Structure of the Partially Reduced c Heme-Oxidized d1 Heme Forms—The crystals at various percentage of the reduced c heme were suitable for x-ray data collection and diffracted at the maximum resolution of 2.70 Å, which is lower than that of the oxidized enzyme (2.15 Å) (3) but comparable with that of the unliganded reduced enzyme (2.90 Å) (4). After the rigid body refinement procedure using NiR-ox as a starting model and the data sets of NiR-15, NiR-50, and NiR-90, and NiR-red crystals, we calculated the first sigmaA maps (2mFo-DFc and mFo-DFc) (Figs. 3, a–c and 4a). We focused our attention on the regions near the prosthetic groups where the wider structural rearrangements had been observed in going from NiR-ox to NiR-red model (4) (Fig. 1). At partial reduction of the c heme, the (2mFo-DFc) maps are always clearly defined and fit well the oxidized conformation (Fig. 3, a–c). The conformation of the residues involved in the movements (loop 56–62 and Tyr10) is superimposable to that of NiR-ox. A peak in the Fourier difference map is observed in between the two propionates of the d1 heme (Figs. 3, a–c and 4a), which was successfully modeled with a water molecule in the NiR-ox model (W564 and W65, in subunit A and B, respectively). Moreover,
one additional Fourier difference peak is observed between the iron atom of the d1 heme and the OH side chain of Tyr10, with a shape comparable with that observed in the NiR-ox structure at the same resolution and identified there as an OH\(^+\) ion (Fig. 3, A–C). No other peaks are visible in the Fourier difference map, which accounts for the good quality of the (2mFo-DFc) map. The presence of the OH\(^+\) strengthens the identity between the partially reduced forms (NiR-15, NiR-50, and NiR-90) and the NiR-ox model.

To truly estimate whether the absence of differences with the NiR-ox structure is significant at the same stage of refinement, i.e. after the rigid body refinement procedure, we have calculated sigmaA maps (2mFo-DFc and mFo-DFc) with NiR-ox as a starting model and the data set of NiR-red (Fig. 4 a). In this case, the oxidized conformation does not fit the (2mFo-DFc) density map. The clear (mFo-DFc) and (2mFo-DFc) maps appear showing the new tracing of the loop 56–62 that corresponds to the reduced conformation. Moreover, a peak in the mFo-DFc map appeared on the side of Tyr10, likely indicating a displaced conformation of the side chain (Fig. 4 a). A Fourier difference peak is also observed near the d1 heme but it is located between the proximal ligand His182 and the iron atom of the d1 heme, far from the position of the OH\(^+\) in NiR-ox. This peak is likely because of a misorientation of the His\(^{182}\) side chain before refinement of the model. Indeed, after refinement by gradient minimization and B-factor refinement, this difference peak has disappeared, and the whole model has slightly moved around this position. Both the loop 56–62 and the region of Tyr10 have been rebuilt. After refinement, the new tracing corresponding to the reduced conformation fit well into the density (Fig. 4b). In the final model, two new hydrogen bonds are present between Thr29 and Gln11, and Ala58 and Gly60, and a significant displacement of the loop 56–62 and Tyr10 with respect to the NiR-ox model can be observed.

The structures of partially reduced enzymes were superimposed on that of the oxidized and of the reduced NiR-Pa, taking into account for the r.m.s. calculation the backbone atoms of the d1 heme domains. Based on this superimposition, the r.m.s. deviations were calculated, and the results are presented in Tables II and III. The deviations to NiR-ox are in the range 0.32–0.56 Å; conversely those to NiR-red are 0.49–1.07 Å, values that are much closer to the 0.69–1.24 Å found for the difference between NiR-red and NiR-ox. By taking into account only the residues involved in the conformational change, in the active site and the ligands of the c and d1 heme groups, the r.m.s. deviations of the partially reduced heme c forms versus NiR-ox are in the range 0.25–0.42, which is significantly different from the 1.4–1.7 found versus NiR-red and for NiR-red versus NiR-ox (Table III). This comparison strengthens the identity of the partially reduced c heme forms to NiR-ox model.

**DISCUSSION**

The present results on the reduction of crystalline NiR-Pa by ascorbate are qualitatively in agreement with the characterization of the reduction reaction previously carried out in solution in the presence of reduced azurin, in which the c heme is reduced more rapidly with a rate constant value \(k_1 = 28 \text{ s}^{-1}\) at room temperature (22), whereas the intramolecular electron transfer is 35-fold slower in the absence of an external ligand acting as an electron acceptor (23, 22). A faster intramolecular electron transfer was detected in the reaction with \(O_2\), where the c heme is oxidized at a rate constant \(k_2 \approx 100 \text{ s}^{-1}\) (8). The quantification of the reduction kinetics in the crystal would demand a complete characterization of the reaction time course, which has not been carried out because it was not the primary motivation of the present study; our goal was to produce and freeze NiR-Pa crystals at different levels of reduction of the c heme to estimate the sequence of structural events involving the d1 heme active site. NiR-Pd crystals have previously been characterized by time-resolved microspectrophotometry during the reduction by dithionite and the reoxidation by nitrite (2). An accurate analysis of the spectra published in Williams et al (2) indicates that, in contrast to the reaction of ascorbate with NiR-Pa, no significant lag was detected between the reduction of the c and the d1 heme. Indeed, a fast intramolecular process has also been observed in a pulse radiolysis study of NiR-Pd in solution; the rate constant of 1400 s\(^{-1}\) at pH 7.0 and room temperature was
assigned to the electron transfer from the c heme to the d₁ heme (25).

The x-ray data presented above on the intermediate oxidation states of NiR-Pa indicate that the structural modification at the level of the c and the d₁ heme observed in going to the fully reduced NiR-red are absent in the partially reduced NiR-15, NiR-50, and NiR-90 models. In light of these results, and in contrast with our previous hypothesis suggesting that the loop 56–62 might move upon reduction of the c heme and trigger the concerted movements at the level of the d₁ active site (4), our present interpretation is that the reduction of the c heme by itself does not trigger the movements of the loop 56–62 and of Tyr¹⁰; these conformational changes appear therefore to be a consequence of additional events in the reduction process that are likely to be the electron transfer to the d₁ heme iron.

From the results in the literature and our studies, the catalytic cycle of NiR-Pa can be summarized by the following Scheme 1.

For step 1, [1]→[2], the c heme of the c-ox/d₁-ox form is rapidly reduced by the electron donor cytochrome c₅₅₁ leading to the c-red/d₁-ox form. The preference of this electron donor for the c heme can originate for two reasons: either the potential of the c-heme is more positive than that of the d₁ heme (26) or the c heme is more accessible than the d₁ heme (27). The latter is confirmed by the three-dimensional structure of NiR-Pa.

For step 2, [2]→[3]→[4], the c-red/d₁-ox form goes through a step involving electron transfer and coupled conformational changes, although the transient c-ox/d₁-red form was not isolated, being immediately followed by the c-red/d₁-red form in the presence of excess reductant. The step [2]→[3] is slow and
might be limited by a slow electron transfer or a slow conformational change involving a reorganization of the coordination site. The d₁ heme reduction might be the trigger of the conformational change. As it is generally agreed that the affinity of oxygenated ligands is much greater for Fe(III) than for Fe(II) (28), addition of one electron at the Fe center of d₁ heme (its reduction) might weaken the Fe-OH bond and provoke the release of the hydroxide ion. This would then induce a series of conformational changes involving the displacement of Tyr₁⁰, likely attracted by the His₃₆₉ to which it is hydrogen-bonded. Indeed, the subunit B of the unliganded NiR-red (4), where the d₁ heme is partly oxidized, presents a conformation close to that proposed above because for Tyr₆₄-OH has been suggested to depend on the close proximity of basic residues (30). However, for NiR-Pa an altered kinetic behavior would have been expected for the mutants Y₁₀₉F and Y₁₀₉N, in which the hydrogen bond to the hydroxide should not be formed (22).

For step 3, [4]→[5]→[2], the c-red/d₁-red form binds nitrite and reduces it to nitric oxide. In the process, the d₁ heme becomes oxidized. At low pH, the affinity of the d₁-oxidized heme for NO is low (< 1 mM) (9), and NO is therefore released ([5]→[2]), leading to the c-red/d₁-ox free enzyme, ready to initiate the next cycle. When the pH is basic, NO remains bound to the c-red/d₁-ox enzyme long enough to lead in the presence of reductants to the stable c-red/d₁-red-NO form which is inactive and essentially “trapped” (10). The protonation state of the active site histidine(s) (His₃₆₉ and His₃₂₇) might be responsible of the high affinity of NO for the enzyme at basic pH.

**CONCLUSION**

An intriguing question is why a conformational change occurs during catalysis. It is first of all clear that the structure of the reduced enzymes from NiR-Pa and NiR-Pd are very similar at the level of the c heme, including the conformation of the loop 55–62 of the c domain. This conformation is also that found in cyto-

### TABLE II

The r.m.s. deviation for the c heme domain (residues 30–115) and the d₁ heme domain (residues 150–536) calculated on the backbone atoms of NiR-ox or NiR-red, respectively, after superimposition of the d₁ heme domain of monomer A.

| NiR-ox | 15% | 50% | 90% | NiR-red |
|--------|-----|-----|-----|---------|
| c Heme domain | Monomer A | 0.33 | 0.40 | 0.40 | 1.24 |
| d₁ Heme domain | Monomer A | 0.15 | 0.15 | 0.16 | 0.21 |
| NiR-red | 1.07 | 1.05 | 1.05 | |
| c Heme domain | Monomer A | 0.17 | 0.17 | 0.18 | |
| d₁ Heme domain | Monomer A | 0.49 | 0.49 | 0.57 | |

### TABLE III

The r.m.s. deviation of the atoms (258 non-hydrogen atoms) of the residues involved in the conformational change, in the active site and ligand of the c and d₁ heme (residues 9–13, 54–64, His₃₂₇ and His₃₆₉, Met₅₁₉, His₃₁₉, c heme and d₁ heme) of partially reduced NiRs (15, 50, and 90% c heme reduced) and the fully reduced c heme, and NiR-ox or NiR-red, respectively, after superimposition of the d₁ heme domain of monomer A.

| NiR-ox | 15% | 50% | 90% | NiR-red |
|--------|-----|-----|-----|---------|
| Active site | Monomer A | 0.25 | 0.29 | 0.29 | 1.74 |
| Monomer B | 0.29 | 0.34 | 0.42 | 1.48 |
| NiR-red | 1.65 | 1.64 | 1.65 | |
| Active site | Monomer A | 1.44 | 1.44 | 1.44 | |
| Monomer B | 0.49 | 0.49 | 0.57 | |

**SCHEME 1. Catalytic cycle of NiR-Pa.** Step 1, [1]→[2]: bimolecular 1.5 × 10⁶ M⁻¹ s⁻¹ with Azurin as electron donor (23); monomolecular 28 s⁻¹ with Azurin in excess (22). Step 2, [2]→[3]: 0.8–1.0 s⁻¹ intramolecular electron transfer from c heme to d₁ heme (10, 22, 23). Step 3, [3]→[4]: as slow as step 2. Step 7, [6]→[7]: faster than step 1 (10). [1], characterized by microspectrophotometry and crystal structure; [2], characterized microspectrophotometry and crystal structure; [3], transient in the catalytic cycle; [4], characterized by microspectrophotometry and crystal structure; [5], transient; [6], transient in the cycle at pH > 7; [7], NO complex characterized by microspectrophotometry and crystal structure (complex with NO blocked at basic pH). Crystal forms in the reduced conformation (d₁ heme site open) are cross-shaded, whereas forms in the oxidized conformation are straight-shaded.
enzymes to make the d1 heme inaccessible. In both these enzymes, the inaccessibility of the heme Fe in the d1 site might be assured either by direct coordination of Tyr\(^{25}\) in NiR-Pd or by the intervention of OH\(^{-}/\text{Tyr}^{10}\) in NiR-Pa. Some analogies in the structure of NiR active site and eukaryote cytochrome c oxidase might be envisaged, as in both cases a Tyr stabilizes an oxygenated ligand (OH\(^{-}\) and O\(_2\)) coordinated at the Fe of the heme (d1 and a3) (24). In cytochrome oxidase, nevertheless, it seems that the protonation state of the Tyr triggers the shift to a catalytically active state of the enzyme, whereas in NiR the role of Tyr seems to protect the active site when the enzyme does not turn over and is, in this respect, somewhat analogous to the Cu\(_{B}\) of cytochrome c oxidase.

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