THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 271, No. 38, Issue of September 20, pp. 23191–23196, 1996
Printed in U.S.A.

Redox Properties of Cytochrome *c* Nitrite Reductase from *Desulfovibrio desulfuricans* ATCC 27774*

(Received for publication, May 9, 1996, and in revised form, July 11, 1996)

Cristina Costa‡, José J. G. Moura‡, Isabel Moura‡¶, Yaning Wang§ and Boi Hanh Huynh§¶

From the ‡Departamento de Química and Centro de Química Fina e Biotecnologia, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2825 Monte da Caparica, Portugal and §Department of Physics, Emory University, Atlanta, Georgia 30322

The dissimilatory nitrite reductase from *Desulfovibrio desulfuricans* ATCC 27774 catalyzes the reduction of nitrite to ammonia. Previous spectroscopic investigation revealed that it is a hexaheme cytochrome containing one high spin ferric heme and five low spin ferric hemes in the oxidized enzyme. The current study uses the high resolution of Mössbauer spectroscopy to obtain redox properties of the six heme groups. Correlating the Mössbauer findings with the EPR data reveals the pairwise spin-spin coupling among four of the heme groups. The other two hemes are found to be magnetically isolated. Reduction with dithionite and reaction with CO further indicate that only the high spin heme is capable of binding small exogenous ligands. These results confirm our previous finding that *Desulfovibrio desulfuricans* nitrite reductase contains six heme groups and that the high spin ferric heme is the substrate and inhibitor binding site.

The dissimilatory reduction of nitrite to ammonia is a common occurrence in microorganisms. It occurs in microaerophiles, such as *Campylobacter sputorum* (1), in several facultative anaerobes, including the *Vibrio* (2, 3) and *Klebsiella* species (4), and in anaerobes such as *Clostridium perfringens* (5), *Clostridium butyricum* (6), *Wolinella succinogens* (7), and *Desulfovibrio desulfuricans* (8). All these organisms share a common presence of a cytochrome that contains multiple *c*-type hemes and is capable of catalyzing the six-electron reduction of nitrite to ammonia.

The cytochrome *c* nitrite reductase from *D. desulfuricans* ATCC 27774 is a membrane-bound protein and has been purified to electrophoretic homogeneity (7). The purified enzyme was reported to be composed of a single polypeptide chain having a molecular weight of 66,000, as judged by sodium dodecyl sulfate gel electrophoresis (7). The enzyme exhibits an optical spectrum typical of *c*-type cytochromes and was reported to contain six *c*-type hemes per 66 kDa based on the extinction coefficient and iron content determination (7). Mössbauer and EPR spectroscopies were used to characterize the heme prosthetic groups (9). At pH 7.6, the as-isolated *D. desulfuricans* nitrite reductase exhibits a complex EPR spectrum consisting of a low spin ferric heme signal at g = 2.96, 2.28, and 1.50 with additional broad resonances at several low field regions (g = 3.92 and 4.8), indicative of spin-spin interaction among the heme groups. EPR redox titration revealed yet another low spin ferric heme signal at g = 3.2 and 2.14 and the presence of a high spin ferric heme (9). Definitive evidence for the presence of six heme groups, however, was provided by the Mössbauer data. The strong field Mössbauer spectra were shown to be superpositions of six spectral components of equal intensity with distinct hyperfine parameters corresponding to six heme groups. One of the heme groups is high spin ferric (S = 5/2) and the others are low spin (S = 1/2) with the following g_{max} values: 3.6, 3.5, 3.2, 3.0, and 2.96 (9). Consistent with the EPR findings, the weak field Mössbauer data show weak magnetic spin-spin interaction between some of the heme groups. Mössbauer studies of a nitrite-reacted sample further suggested that the high spin heme is the substrate binding site.

In this article, we report a detailed redox titration study of the *D. desulfuricans* nitrite reductase monitored by Mössbauer spectroscopy. The unique capability of Mössbauer spectroscopy to detect and distinguish all six heme groups in this enzyme makes it possible to quantify the extent of reduction of each heme group during the titration and therefore allows us to determine the midpoint redox potential for each heme group, a task that is difficult or impossible to achieve using other spectroscopic techniques. The results of this study also provide new information leading to a pairwise assignment for the magnetic heme-heme interactions. In correlation with our current results, implication of the recent finding based on sequence analysis that cytochrome c_{552} from *Escherichia coli*, an ammonia-forming nitrite reductase, is a tetraheme cytochrome (10) is discussed.

MATERIALS AND METHODS

Growth of the Organism and Purification of the D. desulfuricans Nitrite Reductase—D. desulfuricans ATCC 27774 was grown in a medium as described by Liu and Peck (8). Nitrate, rather than sulfate, was used as a terminal electron acceptor to promote the production of nitrite reductase. For the growth of isotopically labeled cells, 400 mg of ^{57}Fe (95% enrichment; Advanced Materials & Technology) was first dissolved in H_2SO_4, then in HCl, neutralized, and added to 400 ml of media. In a typical preparation of the crude extract, 350 g of cells was suspended in 1 liter of 10 mM Tris-HCl buffer (pH 7.6) and ruptured in a French press at 9,000 psi under a nitrogen atmosphere. The extract was centrifuged at 19,000 × *g* for 30 min and then at 180,000 × *g* for 75 min. The pellets obtained in the latter centrifugation contained the cell membranes that were used for purification of the nitrite reductase. The enzyme was purified by the method described previously (8).

Spectroscopic Instrumentation—Optical spectra were recorded on a Shimadzu UV 265 FS spectrophotometer. EPR spectra were recorded on a Brücker ER-200-D-SRC spectrometer equipped with an Oxford Instruments ESR-9 continuous flow cryostat. Standard transmission Mössbauer spectra were recorded with a 50-mCi ^{57}Co(Rh) source driven by a Doppler velocity transducer operating at the constant acceleration mode. Both the weak and strong field Mössbauer spectrometers have been described elsewhere (11). The velocity scale was calibrated using room temperature Mössbauer spectra of a metallic iron foil. The zero velocity is referred to the centroid of these spectra.

Oxidation-Reduction Titration—Oxidation-reduction titrations were

* This work was supported in part by National Institutes of Health Grants GM 47295 (to B. H. H.) and GM 39803 (to Prof. Jean LeGall) and by Grants Praxis/2/2.1/QUI/3/94 and STRDA/BIO/359 (to I. M.) and ERBCHRXT CT 920014 (to J. J. G. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed.

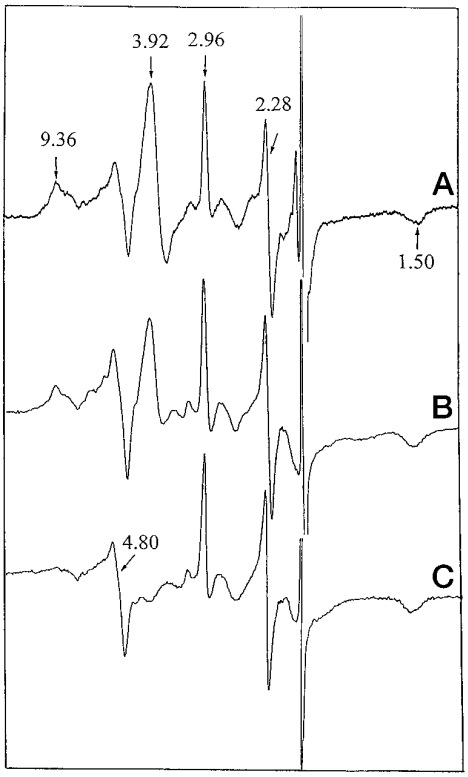

FIG. 1. **EPR spectra of nitrite reductase from *D. desulfuricans* ATCC 27774.** *A,* enzyme in the as-isolated form; *B,* reoxidation by air of a sample reduced under hydrogen atmosphere in the presence of catalytic amounts of hydrogenase; *C,* reoxidation by air of a sample reduced by dithionite. Experimental conditions: microwave frequency, 9.43 GHz; microwave power, 2 mW; temperature, 9 K; modulation amplitude, 1 mT.

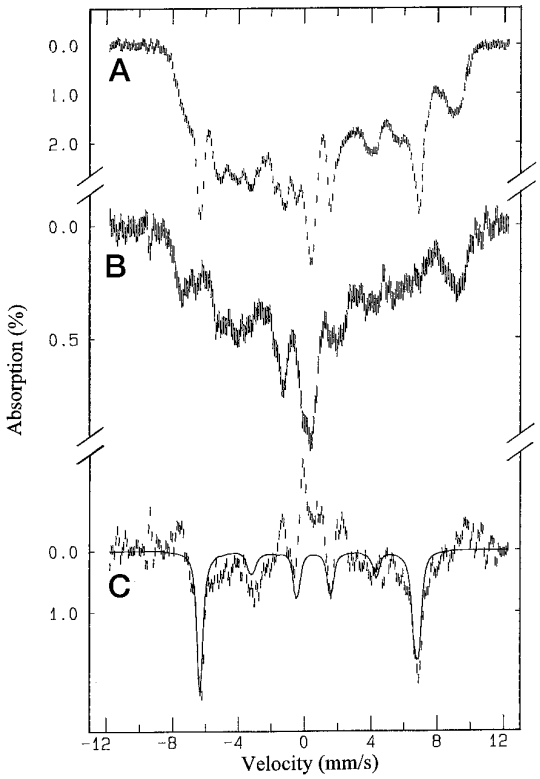

FIG. 2. **Mössbauer spectra of *D. desulfuricans* nitrite reductase.** *A,* as-isolated enzyme; *B,* reoxidized, dithionite-reduced enzyme; *C,* difference spectrum of *A* and *B*. The spectra were recorded at 4.2 K with a magnetic field of 8 T applied parallel to the γ beam. *Solid line* on *C*, theoretical simulation of the high spin ferric heme using the parameters determined previously (9).

carried out in the presence of dye mediators in an anaerobic apparatus similar to that described by Dutton (12), modified to facilitate the simultaneous preparation of Mössbauer and EPR samples at poised redox potentials.

RESULTS AND DISCUSSION

Oxidation-Reduction Cycles—The *D. desulfuricans* nitrite reductase can be fully reduced under a hydrogen atmosphere in the presence of hydrogenase or by a chemical reductant such as sodium dithionite or zinc-reduced methyl viologen. On reoxidation, the EPR and Mössbauer data of the reoxidized enzyme indicate that the reoxidized state of the enzyme depends on the reductant used. Fig. 1 shows the EPR spectra of the reoxidized forms of the enzyme after its reduction with the hydrogen-hydrogenase system (spectrum *B*) and with sodium dithionite (spectrum *C*). For comparison, the EPR spectrum of the as-isolated enzyme is also shown in Fig. 1 (spectrum *A*). It is obvious that spectra *A* and *B* are very similar, indicating that the redox cycle using the hydrogen-hydrogenase reduction system does not affect the heme environments, including the heme-heme interactions. However, reduction with dithionite appears to have altered some of the heme environments; the intense and broad resonance at the g = 3.9 region is absent in Fig. 1, spectrum *C*. To identify the origin of this difference, Mössbauer studies were performed on a dithionite-reduced and then reoxidized sample. (The reoxidized form of a hydrogen- and hydrogenase-reduced sample exhibits Mössbauer spectra identical to those of the as-isolated enzyme (data not shown), consistent with the EPR result.)

Fig. 2 compares the Mössbauer spectrum of the reoxidized dithionite-reduced enzyme (spectrum *B*) with that of the as-isolated enzyme (spectrum *A*). The data were recorded at 4.2 K and in the presence of a strong external applied field of 8 T,[1] which is used to decouple the magnetic heme-heme interactions. The two sharp resonances observed in spectrum *A* at around ± 6.5 mm/s, previously assigned to the high spin ferric heme (9), are absent in spectrum *B*, demonstrating unambiguously that the high spin ferric heme has been affected by the reduction of dithionite. To obtain a more quantitative analysis, a difference spectrum between spectra *A* and *B* is taken and is presented in Fig. 2, spectrum *C*. In such a difference spectrum, the spectral contribution from the unaltered heme groups is canceled, and the observed difference therefore results from the altered heme group(s). Spectrum *C* indicates that only one heme is affected by the reduction-oxidation cycle, and it can be identified as the high spin ferric heme. To illustrate this point, a theoretical simulation using the parameters determined previously (9) for the high spin ferric heme is plotted over the difference spectrum of Fig. 2*C* as a *solid line*. The simulated spectrum agrees very well with the six downward-pointing sharp resonances, indicating unambiguously that the high spin ferric heme has indeed been changed on dithionite reduction. A possible explanation for this observation is that, on dithionite reduction, degraded products of the reductant may bind to the reduced heme, which was initially a high spin ferric heme in the oxidized enzyme, turning it into a low spin ferrous heme. On reoxidation, the degraded product remains bound to the heme, preventing it from reoxidizing. The presence of such a ferrous heme in the reoxidized enzyme is clearly visible at the central region of the difference spectrum as upward-pointing resonances (Fig. 2*C*). Such an explanation is quite plausible, since the high spin ferric heme has been shown to be the

[1] The abbreviation used is: T, tesla.

substrate binding site and complexes with NO after treatment with nitrite (9). Correlating the absence of the broad g = 3.92 EPR signal together with the observed altered state of the high spin heme, it may be concluded that the high spin ferric heme is involved in the heme-heme interaction, which generates the g = 3.92 signal.

Oxidation-Reduction Titrations—A systematic redox titration study was conducted in a redox potential range of +160 to −420 mV. At each poised redox potential, both Mössbauer and EPR samples were prepared to correlate results obtained by the two techniques. The commonly used biological reductant dithionite was not used in this study, since it could alter the ligation state of the high spin ferric heme. Instead, zinc-reduced methylviologen was used for the redox range from +160 to −340 mV, and the −420-mV sample was prepared by the use of the hydrogen and hydrogenase reducing system. The EPR redox titration data have been reported earlier (9). However, due to the presence of heme-heme interactions in this enzyme and the fact that EPR intensities of low spin ferric species with large g_{max} values are intrinsically weak (13), many of the individual heme EPR signals are not observable, and a complete picture of the redox behavior of the heme groups was not obtained through EPR measurements. In contrast, most of the information on the redox properties of each heme group can be extracted from the Mössbauer data, and the results are presented here.

To resolve the spectral contributions from all six heme groups and to decouple the heme-heme interactions, the Mössbauer data were recorded at 4.2 K with a parallel applied field of 8 T. Fig. 3 shows the Mössbauer spectra of *D. desulfuricans* nitrite reductase as a function of the poised redox potential. To estimate the percent reduction of each heme group, a spectral differential method similar to that described in the previous section is applied. The Mössbauer spectrum of the as-isolated oxidized enzyme was used as a reference spectrum, and difference spectra between the reference spectrum and spectra of samples poised at different potentials were prepared. In these prepared spectra, the spectral contributions from the oxidized heme groups are canceled, and only the reduced heme groups will show up as differences. The identification of the individual heme group that gets reduced and its percent reduction can then be estimated by comparing the prepared difference spectra with theoretical simulations using the hyperfine parameters determined previously (9). At low potentials, when several heme groups are reduced, difference spectra between samples poised at consecutive potentials are also prepared in an effort to further simplify the situation. This is obviously a very tedious operation and requires many iterations to arrive at a satisfactory estimation. The results of such an analysis are listed in Table I, and the *solid lines* plotted over the experimental spectra in Fig. 3 are theoretical simulations using these estimated percent reductions. The agreement between theory and experiment is reasonable but not perfect. This is partly due to the fact that the theoretical simulations of the oxidized individual heme groups are not perfect (for example, see Ref. 9). Such imperfection undoubtedly could introduce uncertainties in our estimation. Also, it is important to point out that after several operations of spectral differentiation, the statistical errors are inevitably increased. However, these uncertainties should not affect our conclusion on the order in which the reduction of the heme groups occurs as the potential is decreasing, and we believe that the midpoint redox potentials estimated for the heme groups, presented below, are reasonable.

For the high spin ferric heme and the two low spin ferric hemes with g_{max} values of 2.96 and 3.5, sufficient data points have been obtained to allow an estimate of their midpoint redox

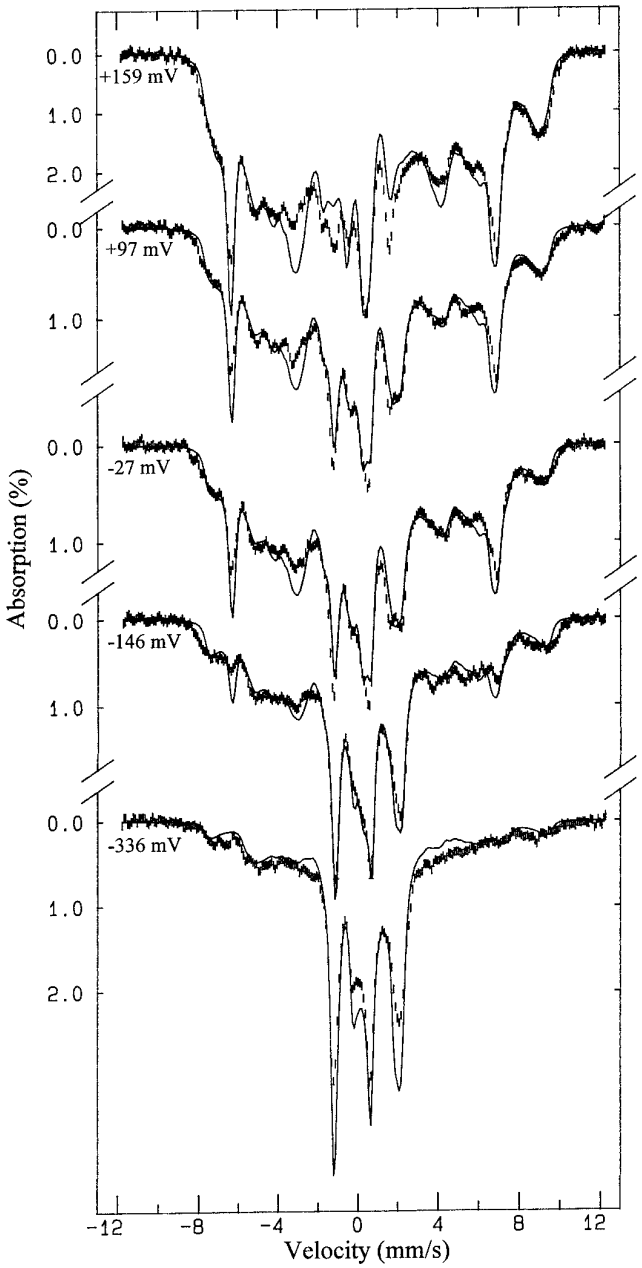

FIG. 3. **Mössbauer spectra of *D. desulfuricans* nitrite reductase as a function of the poised redox potentials at pH 7.6.** The spectra were recorded at 4.2 K with a parallel applied field of 8 T.

potentials by fitting the data to Nernst equations. The percent reductions of these three heme groups as a function of the poised potential are shown in Fig. 4, and the least-squares fits of three independent Nernst equations to the data are plotted as *solid lines*. Their estimated midpoint redox potentials are listed in Table I. The low spin ferric heme with g_{max} = 3.5 was found to have a very positive midpoint redox potential at about +150 mV. A redox titration study using optical spectroscopy (14) has revealed the existence of a very positive redox transition (>+100 mV) for this enzyme. However, the optical method was unable to identify the heme that is responsible for the transition. The current Mössbauer study confirms the optical measurement and identifies the g_{max} = 3.5 heme as the high potential heme.

The midpoint redox potential of the g_{max} = 3.0 heme was found to be relatively low. At a redox potential of −336 mV, about 60% of this heme is reduced. At −420 mV, no oxidizing

TABLE I

Percent reductions of the heme groups in D. desulfuricans nitrite reductase as functions of the poised redox potential and estimated midpoint redox potentials for the heme groups

Mössbauer spectra of low spin ferric hemes are intimately correlated with their EPR g values. Except for hemes 2 and 6, the g_{max} values were estimated from the Mössbauer data (9).

Potential	Heme 1, g_{max} = 3.5	Heme 2, g_{max} = 2.96	Heme 3, high spin	Heme 4, g_{max} = 3.0	Heme 5, g_{max} = 3.6	Heme 6, g_{max} = 3.2
mV						
+159	66					
+97	78					
+69	78		6			
−27	100	27	48			
−36	100	12	18			
−95	100	54	36			
−146	100	78	84			
−174	100	48	72			
−318	100	100	100	36		
−336	100	100	100	60	27	
−420	100	100	100	100	76	64
E'_0 (mV)	+150	−50	−80	−320	−400	−480

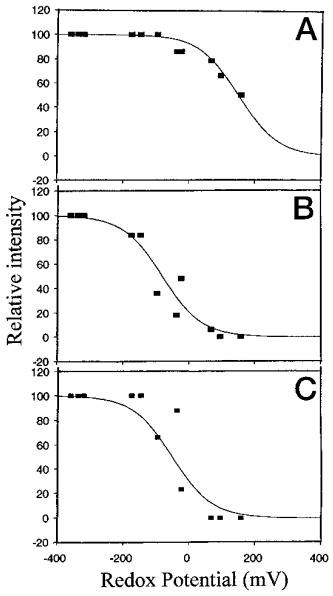

FIG. 4. **Percent reduction of three heme groups in *D desulfuricans* nitrite reductase as determined by Mössbauer spectroscopy.** *A,* g_{max} = 3.5 heme; *B,* high spin ferric heme; *C,* g_{max} = 2.96 heme. *Solid lines,* least squares fits of independent Nernst equations to the data; the resulted midpoint redox potentials are listed in Table I.

component of this heme was detected. Using these experimental results, the midpoint redox potential of this heme is estimated to be approximately −320 mV. The other two low spin ferric hemes, with g_{max} = 3.6 and 3.2, were found to have very low midpoint potentials and remained mostly in the oxidized states even at a potential of −336 mV. However, their midpoint redox potentials can be estimated from the −420-mV sample, which indicates that approximately 24 and 36%, respectively, of the g_{max} = 3.6 and 3.2 hemes remained in their oxidized state. These data allow us to roughly estimate the midpoint redox potentials of the g_{max} = 3.6 and 3.2 hemes to be approximately −400 and −480 mV, respectively.

Pairwise Spin Coupling of the Heme Groups—The fact that the heme groups in the as-isolated *D. desulfuricans* nitrite reductase are magnetically coupled has been firmly established by the Mössbauer spectroscopic study conducted in the presence of a weak applied field and low temperature (9). Correlating the current redox titration study with the previously reported EPR measurements (9) makes it possible to assign the heme groups that are involved in the spin coupling and to reveal the pairing between these coupled hemes.

It is obvious that the g_{max} = 2.96 heme must be magnetically isolated, since its EPR signal is observed in the native enzyme (9). The other magnetically isolated heme is the g_{max} = 3.5 heme. This conclusion is reached by noticing that this heme has a very high redox potential, about 200 mV above that of the next heme, with a lower midpoint potential, and that during the reductive titration at a relatively high redox potential range in which only this heme goes through an oxidation-reduction transition, little alteration is observed in the corresponding EPR spectra. Consequently, this heme must be magnetically isolated. Otherwise, new EPR signals should have appeared during the titration, as this heme is reduced to the diamagnetic low spin ferrous state interrupting the spin-spin interaction.

The remaining four hemes, namely the hemes with g_{max} values of 3.6, 3.2, and 3.0 and the high spin ferric heme, could therefore be involved in spin coupling. In the EPR spectrum of the as-isolated enzyme, the broad absorption-type signal at g = 3.92 and the derivative-type signal with zero crossing at g = 4.8 have been attributed to represent spin-spin interactions between the heme groups (9, 15). In the EPR spectra of both the nitrite-reacted enzyme, presented previously (9), and the reoxidized dithionite-reduced enzyme, presented above, the broad signal at g = 3.92 disappears, whereas the derivative signal at g = 4.8 remains, suggesting that these two signals represent two independent interactions. Since the minimum number of heme groups that could form a spin-spin interaction is two, and there are only four heme groups available for the two independent interactions, these four heme groups must be coupled in pairs.

One of the two hemes that is involved in the coupling that generated the g = 3.92 signal has been identified unambiguously as the high spin ferric heme (see above and Ref. 9). The other heme of this coupled pair is proposed to be the g_{max} = 3.2 heme. This assignment is based on the observation that, in the EPR spectrum of the nitrite-reacted sample, the disappearance of the g = 3.92 signal is accompanied by the appearance of a new low spin ferric EPR signal at g = 3.2 and 2.14 (9). The other two hemes with g_{max} values of 3.6 and 3.0 are therefore assigned to the other coupled pair and are associated with the g = 4.8 signal. This assignment is consistent with the observation that, during the course of redox titration, no new signals at g = 3.0 and 3.6 are detected. The signal at g = 3.6 is not expected to be observed, since its intensity is about 2 orders of magnitude weaker than signals at around g = 3.0 (13). The failure to detect a signal at g = 3.0 can be explained by the proposed coupling assignment in conjunction with the fact that the g_{max} = 3.0 heme has a higher redox potential than that of

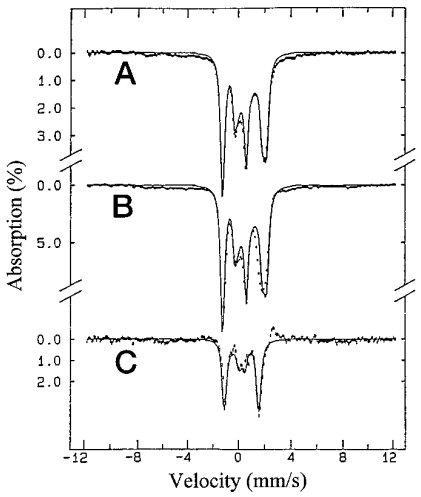

FIG. 5. **Mössbauer spectra of *D desulfuricans* nitrite reductase.** *A,* enzyme reduced under hydrogen atmosphere in the presence of hydrogenase; *B,* hydrogen-reduced enzyme of *A* reacted with CO; *C,* difference spectrum (*A* − 85.4% of *B*) representing the CO-bound heme (see text). The spectra were recorded at 4.2 K with a parallel applied field of 8 T. *Solid lines* in *A* and *B*, same theoretical simulation using the parameters obtained for the reduced enzyme at zero field (ΔE_Q = 1.10 mm/s; δ = 0.45 mm/s) and assuming diamagnetism. *Solid line* in *C*, theoretical simulation using the parameters reported for hemoglobin-CO (17).

the g_{max} = 3.6 heme. Consequently, during the redox titration, the g_{max} = 3.0 heme is either reduced, which is EPR silent, or spin coupled to the g_{max} = 3.6 heme showing the derivative-type g = 4.8 signal. The couplings of these four heme groups can be completely decoupled at applied magnetic fields above 4 T (9), indicating that the coupling strength is very weak (less than 1 cm^{-1}) and dipolar in nature.

Reduction of D. desulfuricans Nitrite Reductase with H_2-Hydrogenase—In Fig. 5, spectrum *A* is the Mössbauer spectrum of enzyme reduced by the hydrogen- and hydrogenase-reducing system. The data were recorded at 4.2 K and in the presence of a 8-T field applied parallel to the γ beam. Data recorded in the absence of a magnetic field show a single quadrupole doublet with parameters (ΔE_Q = 1.10 ± 0.03 mm/s; δ = 0.45 ± 0.02 mm/s) typical of low spin ferrous hemes (data not shown). The *solid line* overlaid on Fig. 5, spectrum *A*, is a theoretical simulation using the parameters obtained at zero field and assuming diamagnetism. The area of the solid line is normalized to 90% of that of the experimental spectrum. A very good agreement between the theory and experimental data is observed, indicating that the reduced heme groups are indeed diamagnetic, as expected for low spin ferrous hemes. The remaining 10% of the absorption can be attributed to the oxidized g_{max} = 3.6 (4%) and 3.2 (6%) hemes, the two heme groups with the lowest midpoint potentials.

In proteins, reduction of a high spin ferric heme is generally expected to yield a high spin ferrous heme, as observed for the heme groups in hemoglobin and myoglobins. In contrast, the Mössbauer data presented above show that, on reduction, the spin states of all the heme groups in the *D. desulfuricans* nitrite reductase are low spin, including the high spin ferric heme. Since high spin Fe ions are believed to be either five coordinated or with a weak sixth ligand and low spin Fe ions are generally six coordinated with very few exceptions (16), the above observation suggests that on reduction the high spin ferric heme may have picked up a sixth ligand, most probably a nearby amino acid residue at the distal side of the heme group.

Reaction of Carbon Monoxide with Reduced Nitrite Reductase—The active state of the *D. desulfuricans* nitrite reductase is the reduced form of the enzyme, and its activity can be inhibited by CO. To investigate the effect of CO on the reduced heme groups, we treated the reduced enzyme with CO. Fig. 5*B* shows a Mössbauer spectrum of the CO-reacted enzyme recorded at 4.2 K with a parallel field of 8 T. At first glance, spectrum *B* appears to be similar to spectrum *A*. To show that the two spectra are in fact different, the theoretical simulation shown in Fig. 5*A* (*solid line*) is overlaid onto spectrum *B*. The difference is obvious and can be attributed to the binding of CO to *one* of the reduced low spin ferrous hemes (see below). To illustrate that only one heme, corresponding to 16.6% of the total Fe absorption, is affected by the reaction with CO, we subtracted from spectrum *B* 85.4% of spectrum *A*, and the difference spectrum is shown in Fig. 5*C*. This spectrum is also typical of diamagnetic ferrous low spin species but is distinct from that of the reduced nitrite reductase shown in Fig. 5*A*. Interestingly, this difference spectrum (Fig. 5*C*) is found to be very similar to that of the hemoglobin-CO complex. Using the published parameters of hemoglobin-CO (17) (ΔE_Q = 0.36 mm/s; δ = 0.26 mm/s), a theoretical spectrum was simulated and plotted over spectrum *C* as a *solid line*. The agreement between the theoretical and the experimental spectra is excellent. This analysis indicates that the Mössbauer data are consistent with the premise that only one of the reduced heme groups is complexed with CO and the remaining heme groups are not affected. Considering the results obtained from the nitrite-reacted enzyme and the reoxidized dithionite-reduced enzyme, which indicate that only the high spin ferric heme of the as-isolated enzyme can bind small exogenous ligands after reduction, it is reasonable to suggest that the high spin ferric heme is the site that binds CO. Although the high spin ferric heme is reduced to a six-coordinated low spin ferrous state, the binding of the sixth ligand is probably weak so that it can be displaced by small molecules such as CO and NO. Complexation of CO to ferrous heme is a common occurrence for heme enzymes and has also been reported for a similar cytochrome *c* nitrite reductase from *W. succinogens* (18–20). However, binding of CO to two of the heme groups was reported for the *Wolinella* nitrite reductase.

Final Remarks—The current study uses high resolution Mössbauer spectroscopy to obtain redox properties of the six heme groups in the *D. desulfuricans* nitrite reductase. Correlating the Mössbauer findings with the EPR data reveals the pairwise spin-spin coupling among four of the heme groups. The other two hemes are found to be magnetically isolated. Reduction with dithionite and reaction with CO further indicate that only the high spin heme is capable of binding small exogenous ligands. These results confirm our previous finding that *D. desulfuricans* nitrite reductase contains six heme groups and that the high spin ferric heme is the substrate and inhibitor binding site. In this respect, it is interesting to note that a similar cytochrome *c* nitrite reductase (cytochrome c_{552}) isolated from *E. coli*, previously reported as a hexaheme cytochrome (21, 22), was recently suggested to be a tetraheme cytochrome based on the sequence analysis of the *nrfA* structural gene, which shows only four Cys-*X*-*X*-His cytochrome *c* binding motifs with a predicted molecular mass of 54 kDa (10). More recently, it was reported that two forms of a cytochrome *c* nitrite reductase were isolated from the membrane fraction of *Sulfurospirillum deleyianum*, a monomeric form with a molecular mass of 55 kDa and a hetero-oligomeric form composed of two polypeptide chains with molecular masses of 55 and 20 kDa (23). Although the oligomeric form exhibits a EPR spectrum that is almost identical to that of the *D. desulfuricans* nitrite reductase (9), the monomeric form displays a similar EPR

spectrum but with the absence of the derivative-type signal at g = 4.8 (23). Heme content determination further indicates that the monomeric molecule contains only four heme groups per 55 kDa (23). Taking into consideration these new developments together with the current spectroscopic results, it may be possible that *D. desulfuricans* nitrite reductase is a heterooligomer composed of a tetraheme and a diheme subunit. To address this interesting and still open question, applications of modern molecular biology to identify and to sequence the structural gene of the *D. desulfuricans* nitrite reductase have been planned.

Acknowledgments—We thank Dr. Ming Y. Liu and the staff of the Fermentation Plant at the University of Georgia for growing the bacterial cells.

REFERENCES

1. deVries, W., Niekus, H. G. D., Boellaard, M., and Stouthamer, A. H. (1980) *Arch. Microbiol.* **124,** 221–227
2. Liu, M.-C., Bakel, B. W., Liu, M. Y., and Dao, T. N. (1988) *Arch. Biochem. Biophys.* **262,** 259–265
3. Rehr, B., and Klemme, J. H. (1986) *FEMS Microbiol. Lett.* **35,** 325–328
4. Hadjipetro, L. P., and Stouthamer, A. H. (1965) *J. Gen. Microbiol.* **38,** 29–34
5. Hasan, S. M., and Hall, J. B. (1975) *J. Gen. Microbiol.* **87,** 120–128
6. Keith, S. M., MacFarlane, G. T., and Herbert, R. A. (1982) *Arch. Microbiol.* **132,** 62–66
7. Liu, M. C., Liu, M. Y., Payne, W. J., Peck, H. D., and LeGall, J. (1983) *FEMS Microbiol. Lett.* **19,** 201–206
8. Liu, M.-C., and Peck, H. D., Jr. (1981) *J. Biol. Chem.* **256,** 13159–13164
9. Costa, C., Moura, J. J. G., Moura, I., Liu, M. Y., Peck, H. D., Jr., LeGall, J., Wang, Y., and Huynh, B. H. (1990) *J. Biol. Chem.* **265,** 14382–14387
10. Darwin, A., Hussain, H., Griffiths, L., Grove, J., Sambongi, Y., Busby, S., and Cole, J. (1993) *Mol. Microbiol.* **9,** 1255–1265
11. Kretchmar, S. A., Teixeira, M., Huynh, B. H., and Raymond, K. N. (1988) *Biol. Metals* **1,** 26–32
12. Dutton, P. L. (1978) *Methods Enzymol.* **54,** 411–435
13. Aasa, R., and Vänngård, T. (1975) *J. Magn. Reson.* **19,** 308–315
14. Costa, C. (1994) *Caracterização Espectroscópica de Sistemas Hémicos Isolados de Desulfovibrio desulfuricans ATCC 27774, uma Bactéria Redutora de Sulfato com Capacidade Metabólica de Reduzir o Nitrato.* Ph.D thesis, Universidade Nova de Lisboa
15. Andersson, K. K., Lipscomb, J. D., Valentine, M., Münck, E., and Hooper, A. B. (1986) *J. Biol. Chem.* **261,** 1126–1138
16. Nasri, H., Wang, Y., Huynh, B. H., and Scheidt, W. R. (1991) *J. Am. Chem. Soc.* **113,** 717–719
17. Lang, G., and Marshall, W. (1966) *Proc. Phys. Soc.* **87,** 3–34
18. Blackmore, R., Roberton, A. M., and Brittain, T. (1986) *Biochem. J.* **233,** 547–552
19. Blackmore, R., and Brittain, T. (1986) *Biochem. J.* **233,** 553–557
20. Blackmore, R., Gadbsy, P. M. A., Greenwood, C., and Thomson, A. J. (1990) *FEBS Lett.* **264,** 257–262
21. Kajie, S., and Anraku, Y. (1986) *Eur. J. Biochem.* **154,** 457–463
22. Liu, M. C., Liu, M. Y., Payne, W. J., Peck, H. D., Jr., LeGall, J., and DerVartanian, D. V. (1987) *FEBS Lett.* **218,** 227–230
23. Schumacher, W., Hole, H., and Kroneck, P. M. H. (1994) *Biochem. Biophys. Res. Commun.* **205,** 911–916

Redox Properties of Cytochrome *c* Nitrite Reductase from *Desulfovibrio desulfuricans* ATCC 27774

Cristina Costa, José J. G. Moura, Isabel Moura, Yaning Wang and Boi Hanh Huynh

J. Biol. Chem. 1996, 271:23191-23196.
doi: 10.1074/jbc.271.38.23191