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FT-IR analysis of membranes of *Rhodobacter sphaeroides* 2.4.3 grown under microaerobic and denitrifying conditions

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Abstract

Fourier transform infrared spectroscopic analysis of CO binding proteins in *Rhodobacter sphaeroides* reveals the presence of a membrane-bound nitric oxide reductase (Nor). Nor has been clearly distinguished from the cytochrome oxidases by the temperature-dependence of relaxation following photodissociation of the CO complex at cryogenic temperatures. The center frequency and band shape, 1970 cm^{-1} and 20–30 cm^{-1} width at half-peak height, are similar to those reported for resonance Raman spectra of purified *Paracoccus denitrificans* Nor. Additional evidence is presented to indicate this enzyme is part of dissimilatory nitric oxide metabolism and that one of the genes in the *nor* operon required for production of an active Nor is not required for protein assembly or heme incorporation.

Keywords: Fourier transform infrared spectroscopy; Oxidase; Denitrification; (*Rhodobacter sphaeroides*)

1. Introduction

Respiratory bacteria have the capacity to produce a wide array of terminal oxido-reductases during growth. Many aerobic bacteria encode at least two and as many as four oxygen reducing oxidases, each of which probably has a distinct physiological function [1]. In addition to oxygen-reducing enzymes, other types of terminal oxido-reductases can be expressed if alternative electron acceptors are present. For example, denitrifying bacteria, which can utilize nitrate as an alternative electron acceptor, will express multiple nitrogen oxide reductases if nitrate is present and oxygen is limiting [2]. This means that denitrifiers, like most bacteria capable of respiration, have the capacity to express a variety of terminal oxido-reductases.

Many different biochemical and spectroscopic techniques have been used to characterize terminal respiratory oxido-reductases. One of these techniques, Fourier transform infrared spectroscopy (FT-IR), has been very useful for elucidating structural details about the active site of members of the heme–copper family of cytochrome oxidases. By monitoring the vibrational frequency of carbon monoxide (CO) bound to metal centers in the enzyme, information about the local molecular environment of both the high-spin heme and the copper center, which together form the site of oxygen reduction, has been obtained. FT-IR has been used to study the terminal heme–copper oxidases of the bacterium *Rhodobacter sphaeroides* in some detail [3–5]. These

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studies have revealed that there are two oxidases and that their active sites have significant structural differences. The FT-IR spectrum of the aa_3-type oxidase, preferentially expressed at high oxygen concentrations, is very similar to that of the mitochondrial aa_3-type cytochrome oxidase, consistent with their sequence similarity [6]. One unique feature of the *R. sphaeroides* and mitochondrial aa_3-type oxidases is that they have two spectrally distinct conformational states or forms [3,7]. The spectrum of the cbb_3-type oxidase, expressed when oxygen concentrations are low, has only one form which is significantly different from either form of the aa_3-type oxidase, reflecting this enzymes limited sequence similarity to the aa_3-type oxidase. The differences in the active site shown by FT-IR no doubt reflect the different physiological roles of these enzymes.

In addition to the heme–copper oxidases, most strains of *R. sphaeroides* also encode nitric oxide reductase (Nor) [8]. Nor is related to the heme–copper family of cytochrome oxidases [9]. However, instead of a heme–copper binuclear center it appears to have a binuclear center consisting of a heme and a nonheme iron [10,19]. Since FT-IR with CO as a spectroscopic probe has proved effective in studying the active site of the heme–copper oxidases, a similar study has been carried out on membranes of a denitrifying strain of *R. sphaeroides*, strain 2.4.3. Membranes from cells cultured under microaerobic or denitrifying conditions were analyzed. FT-IR spectra of membranes of cells of 2.4.3 cultured in medium unamended with nitrate were similar to the spectrum of membranes of 2.4.1 cells grown under low oxygen, with the predominant spectroscopic features deriving from the cbb_3-type oxidase. However, membranes from cells grown as denitrifiers exhibited additional spectroscopic features which, based on data from this and other studies, is shown to be due to Nor.

2. Materials and methods

2.1. Bacterial strains and growth conditions

R. sphaeroides strain 2.4.3 (ATCC17025) was the wild-type denitrifying strain. Strain 46 is a 2.4.3 derivative in which *norQ* contains a Tn-*lacZ* insertion [11]. The insertion of the Tn has been determined by sequence analysis to be at base 463 of the *norQ* open reading frame. All strains were grown in Sistrom's medium at 30°C with an agitation rate of 225 rpm. Cells grown microaerobically were cultured as previously described [12]. Cells grown in medium without added nitrate were harvested at an optical density, measured at 600 nm, of 0.7. Cells grow in amended medium were harvested at an optical density at 600 nm of about 1.0. Nitrate was added to cultures to a final concentration of 12 mM as the potassium salt.

2.2. Sample preparation

After harvesting the cells by centrifugation, cytoplasmic membranes were prepared exactly as described previously [3]. CO adducts of cytoplasmic membranes for use in FT-IR spectroscopy were generated as previously described [3]. A portion of the sample was pressed between two $CaFl_2$ windows to a desired thickness. Infrared spectra were obtained with a Mattson Sirius 100 FT-IR interferometer at a resolution of 0.5 cm^{-1}. Sample temperatures were maintained at the appropriate temperature with a Lake Shore Cryotronics closed-cycle helium refrigerator. Spectra were obtained in the range of 1800 to 2200 cm^{-1} using a liquid nitrogen cooled indium-atinimide detector. Spectra are presented as difference spectra, in which the spectrum before photolysis (dark) is subtracted from the spectrum obtained during or after photolysis (light). Photolysis was carried out as previously described [3]. Light and dark spectra are an average of 512 scans.

3. Results

3.1. FT-IR analysis of membranes

The FT-IR spectrum of CO bound to inner membranes of microaerobically grown cells is shown in Fig. 1. Since this is a light-minus-dark difference spectrum, the spectroscopic features which point downward are due to CO interacting with its local molecular environment before photolysis and those pointing upward are from photolyzed CO. Two downward pointing features with maximally negative values of 1950 cm^{-1} and 1965 cm^{-1} are present. The absorbance at the lower wave number is from CO

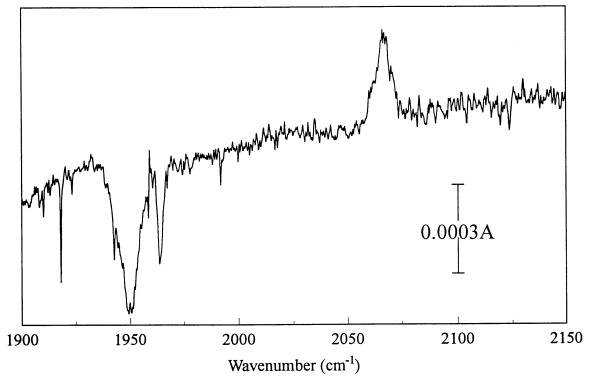

Fig. 1. Light-minus-dark infrared absorbance spectrum of CO adducts of glycerol-extracted inner membranes of cells of *R. sphaeroides* 2.4.3 cultured microaerobically in medium unamended with nitrate. The spectrum was obtained at 12 K. The Fe–CO have minimal values at 1950 cm^{-1} and 1965 cm^{-1}, the Cu–CO has a maximal value at 2065 cm^{-1}.

bound to the ferrous heme of the cbb_3-type oxidase since it matches that seen for the purified cbb_3-type oxidase [4]. The single upward pointing feature with a peak at 2065 cm^{-1} arises from CO that has been photolyzed from the heme and is now bound to the copper located nearby in the binuclear active site of the cbb_3-type oxidase. The 1965 cm^{-1} feature is due to the aa_3-type oxidase [3]. However, the Cu–CO bands associated with the aa_3-type oxidase are not readily distinguished above the noise, due to the small amount of the aa_3-type oxidase in the membranes. The very narrow peak at 1918 cm^{-1} is from residual atmospheric water vapor in the optical path. The spectrum of these microaerobically grown cells of strain 2.4.3 most closely resembles the spectrum of *R. sphaeroides* strain Ga cells grown photosynthetically [3].

The 12 K light-minus-dark difference spectrum of membranes of cells cultured microaerobically with nitrate is shown in Fig. 2A. The Fe–CO region of this spectrum is much broader than comparable regions in the membranes from cells grown without nitrate. The spectrum appears to consist of the 1950 cm^{-1} and 1964 cm^{-1} bands present in the spectrum of membranes from microaerobically cultured cells plus a third, broad Fe–CO absorbance with a maximum negative value at about 1970 cm^{-1}. The Cu–CO region is similar to the same region in the other membranes with only a single absorbance at 2065 cm^{-1}. Given the large amplitude of the 1970 cm^{-1} signal and the high signal/noise ratio of this spectrum, the absence of a second Cu–CO indicates the protein giving rise to the broad Fe–CO is not a heme–copper containing oxidase.

Differences in temperature-dependent relaxation rates after CO photolysis can be used to spectrally distinguish heme–copper oxidases from those which do not contain a Cu–CO binding site. Many photodissociation products are sufficiently stable at 12 K to be observed by absorbance difference spectroscopy. Relaxation back to the ground state occurs rapidly at 180 K. At 120 K the photodissociated state is stable for enzymes with a heme–copper binu-

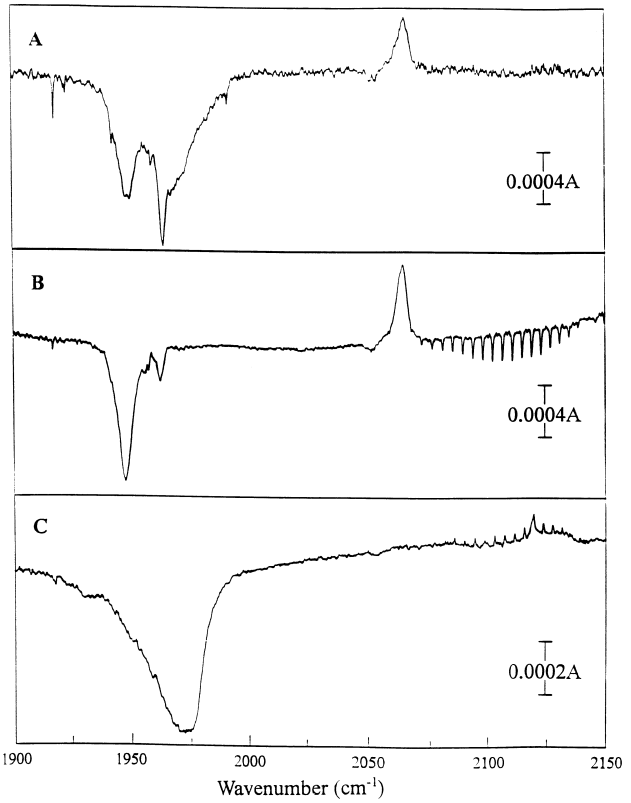

Fig. 2. Light-minus-dark infrared absorbance spectrum of CO adducts of glycerol-extracted inner membranes of cells of *R. sphaeroides* 2.4.3 cultured microaerobically in medium amended with nitrate. The spectrum in panel A was taken at 12 K. For panel B, the same sample used to obtain the spectrum in panel A was raised to 180 K and then lowered to 120 K. A dark spectrum was obtained followed by photolysis for 10 min and a relaxation period of 15 min, at which point a light spectrum was obtained. For panel C, after completion of the light spectrum of the sample in panel B, the temperature was lowered to 12 K. A dark spectrum was obtained, followed by a spectrum taken under continuous photolysis.

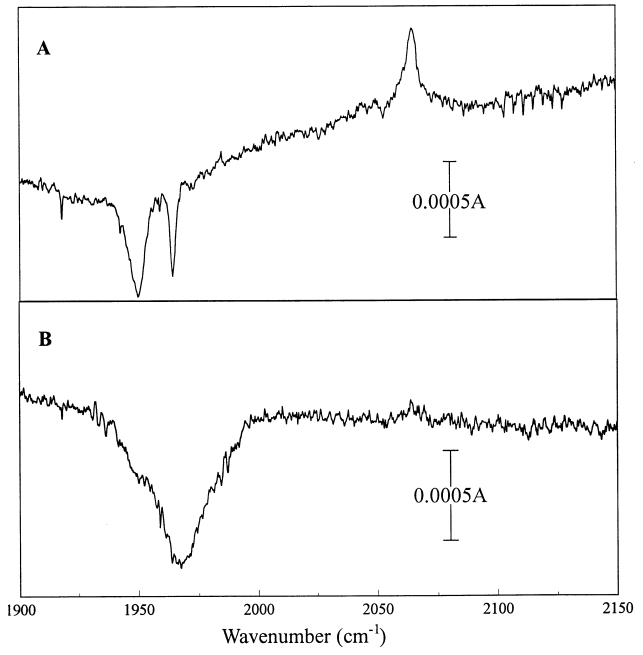

Fig. 3. Light-minus-dark infrared absorbance spectrum of CO adducts of glycerol-extracted inner membranes of NorQ-deficient cells of *R. sphaeroides* 2.4.3 cultured microaerobically in medium unamended with nitrate. For panel A the sample was lowered to 120 K. A dark spectrum was obtained followed by photolysis for 10 min and a relaxation period of 15 min, at which point a light spectrum was obtained. For panel B, after completion of the light spectrum of the sample in panel A, the temperature was lowered to 12 K. A dark spectrum was obtained, followed by a spectrum taken under continuous photolysis.

clear center, but relaxation is rapid for enzymes which lack Cu–CO binding site. Therefore, the sample shown in Fig. 2A was warmed to 180 K to relax all the photodissociable species. Then the sample was cooled to 120 K, a spectrum was measured in the dark, the sample photodissociated and then relaxed for 15 min to allow non-heme–copper enzymes to return to the ground state before recording a light spectrum. The ratio of light/dark spectra at 120 K therefore contained only spectra of the heme–copper oxidases (Fig. 2B). The sample was then cooled to 12 K without a higher temperature relaxation, so the CO remained coordinated as non-photodissociable Cu–CO in the heme–copper oxidases. The spectrum of the non-copper containing enzyme in the membranes was then isolated by measurements at 12 K (Fig. 2C).

The difference spectrum at 12 K contains a single Fe–CO which had a maximum negative value centered around 1972 cm^{-1}. This band is broad, with a width of about 25 cm^{-1} at half-peak height. The band shape may be noise limited but appears to be somewhat asymmetric, suggesting the presence of an additional underlying band. Similar broad bands have been reported for CO complexes in peroxidases and cytochrome *P*-450 [13,14]. There was also a small peak centered at 2120 cm^{-1}. Note that the small positive band at 2120 cm^{-1} has a half-band width of about 2 cm^{-1}, and is clearly distinguished from much narrower surrounding lines that are due to small differences between sample and reference data collections of atmospheric CO contained in the instrument purge air. Similar lines with 0.5 cm^{-1} (resolution-limited) band widths also appear in Fig. 2B. Absorption in the 2120 cm^{-1} region arises from CO interacting weakly with groups in the heme pocket [15]. This indicates the photolyzed CO does not bind to a metal and is consistent with the complete relaxation of the CO bound to the active site of this protein at 120 K.

We also carried out a similar FT-IR analysis of membranes of a strain in which *norQ*, a gene within the *nor* operon of 2.4.3, had been inactivated [11]. This was done to provide evidence that the protein giving rise to the 1970 cm^{-1} peak is specifically involved in nitric oxide (NO) metabolism. It has been observed that when mutants of 2.4.3 which lack Nor activity are cultured in medium unamended with nitrate, genes whose products are involved in dissimilatory NO metabolism, including both Nor and nitrite reductase, are expressed [16]. This is apparently because NO accumulates in these strains resulting in the expression of Nor and other genes. Nor activity can be eliminated in 2.4.3 by insertionally inactivating several genes within the *nor* operon, including *norQ* [11]. Inactivation of the likely *norQ* homolog in *Pseudomonas stutzeri*, which has been designated *nirQ*, also resulted in a loss of Nor activity [17]. However, it was found that this did not prevent expression of Nor. From these results it might be predicted then that proteins involved in dissimilatory NO metabolism, including an inactive Nor, would be expressed in a *norQ* mutant grown in unamended medium. These culture conditions should not result in expression of those portions of the denitrification pathway not involved in NO metabolism, since nitrate was not added to the medium.

Membranes of cells of the *norQ* mutant strain 46 were analyzed using temperature manipulation to separate the spectrum arising from the various CO-binding proteins. The spectrum obtained from subtracting relaxed-photolyzed minus dark spectrum measured at 120 K showed two Fe–CO with maximally negative values at 1950 cm^{-1} and 1964 cm^{-1} and a single Cu–CO with a peak at 2065 cm^{-1} (Fig. 3A). Light-minus-dark difference spectrum of the sample taken at 12 K, following photolysis at 120 K, contained a broad Fe–CO centered at about 1970 cm^{-1} (Fig. 3B). This band has a width of 27 cm^{-1} at half-peak height, similar to the feature in the wild-type membranes. Any peak at 2120 cm^{-1} would be buried in the noise, and as expected, was not observed. The presence of this CO-binding protein in this mutant indicates the protein is required for dissimilatory NO metabolism, and is therefore likely to be Nor.

4. Discussion

The FT-IR spectrum of CO bound to membranes of cells of *R. sphaeroides* 2.4.3 grown in medium unamended with nitrate was nearly identical to that of cells of a *R. sphaeroides* 2.4.1 derivative grown photosynthetically [3]. This demonstrates the predominant oxidase in 2.4.3 under low oxygen was the cbb_3-type oxidase. The limited expression of the aa_3-type oxidase in the membranes of cells used in this study was a result of oxygen being limiting in all the cell cultures, leading to the preferential expression of the cbb_3-type oxidase, consistent with recent studies on regulation of expression of genes encoding the aa_3-type oxidase [18].

Inclusion of nitrate in the medium of wild-type cells induced the expression of a CO-binding protein not previously detected in *R. sphaeroides* strains. This protein does not contain a heme–copper binuclear center, was only expressed in wild-type cells in nitrate amended medium, binds CO through a heme associated iron, since the Fe–CO is susceptible to photolysis, and is membrane bound because the preparative procedures should have removed any proteins not tightly associated with the membrane. Recent work strongly suggests this CO binding protein is Nor. Characterization of Nor from *P. denitrificans* [19] has shown that it contains a low-spin heme *b*, low-spin heme *c*, high-spin heme *b* and non-heme iron in the ratio of 1:1:1:1, as predicted from sequence analysis [9]. The absence of EPR signals arising from the high-spin heme *b* and non-heme iron suggests they are in close proximity, as expected. Further characterization of this enzyme from *P. denitrificans* using resonance Raman spectroscopy showed that CO binds to the high-spin heme in the binuclear center, converting it to a six-coordinate, low-spin form [20]. Significantly, resonance Raman measurement of the vibrational frequency of the ν_{C-O} stretching mode, the same mode monitored in the FT-IR experiments, showed the mode had a frequency of 1970 cm^{-1}. This is the exactly the same value as determined by FT-IR for the CO-binding protein uniquely present in the membranes of the nitrate-grown cells. It seems unlikely the CO binding characterized in this study is anything other than Nor. The 1970 cm^{-1} is significantly different from the values measured for heme–copper oxidases or other heme-containing proteins.

One puzzling feature of the Nor spectrum is the lack of formation of the metal–CO complex formed after photolysis (Fig. 2B,C). It seems reasonable to expect that the photolyzed CO could bind to the non-heme iron located at the active site in Nor, analogous to the binding of CO to Cu_B in the active site of heme–copper oxidases, since the heme iron and non-heme iron in Nor are close enough to be antiferromagnetically coupled [19]. However, the absence of an upward pointing feature in the Fe–CO region along with the rapid relaxation of the photolyzed CO at 120 K are inconsistent with photolyzed CO interacting with a nearby metal center. This suggests that either there is no non-heme iron adjacent to the high-spin heme, which seems unlikely [10,19], or that the non-heme iron cannot bind CO. An explanation for why CO might not bind to the non-heme iron may lie in the reaction mechanism of Nor. Nor must reduce two NO molecules to produce one N_2O. It has been suggested that to catalyze the formation of the N–N bond the active site of Nor binds two NO molecules, one to the heme iron and the second to the non-heme iron [20]. If this model is correct it also seems likely that the active site could bind two molecules of CO, one at each metal center. Only the CO bound to the heme would be susceptible to photolysis and consequently detectable in a light-minus-

dark difference spectrum. Since CO bound to the non-heme iron would remain there after photolysis, this would prevent the photolyzed CO from binding to the non-heme iron. The photolyzed CO would then interact with the amino acids which form the binding site pocket, as observed in Fig. 2C. Therefore, the FT-IR results provide support for the reaction mechanism that substrate may bind to both heme and non-heme iron in Nor. In the heme–copper oxidases the heme and the copper centers do not bind CO at the same time. The copper center can only bind CO photolyzed from the heme.

Further confirmation that Nor gives rise to the 1972 cm^{-1} band was provided by the detection of this band in NorQ-deficient cells. Since no nitrate was added to the medium in which these cells were cultured, the protein giving rise to this band must be involved in dissimilatory NO metabolism. In addition, this result has implications for the physiological function of NorQ. The Fe–CO signal of the non-oxidase component in the NorQ-deficient membranes was very similar to the that observed in wild-type membranes of cells grown in medium amended with nitrate. There was a slight increase in the wave number of the maximal negative value of the feature in the NorQ-deficient cells, but the band width at half-maximum peak height was very similar in both sets of membranes (Fig. 2C, Fig. 3B). This indicates that there had not been a significant disruption of the local molecular environment of the CO-binding site in the Nor-deficient strain. Therefore, the role of NorQ can not be in the assembly or insertion of heme into Nor. It is possible that NorQ may play a role in insertion of non-heme iron but since the non-heme iron is not detectable its presence can not be definitively assessed.

FT-IR analysis of membranes of various strains of *R. sphaeroides* has now detected CO-binding by three different proteins. Two of these are heme–copper oxidases [3] and the third is likely to be a member of this family as well. Even though these proteins are all members of the same protein superfamily, the sequence identity between them is relatively low, with pairwise alignments having identities less than 25% (data not shown). This sequence dissimilarity is manifested in the FT-IR difference spectrum. CO-binding to the aa_3-type oxidase of *R. sphaeroides* revealed that it is present in two spectrally distinct, pH-dependent conformational states, referred to as the α and β forms [3]. The cbb_3-type oxidase has a single Fe–CO which is most similar in shape and center frequency to the β form of the aa_3-type oxidase, but the Cu–CO produced upon photolysis is most similar in shape and center frequency to the Cu–CO of the α form. The differences of the FT-IR spectrum of the aa_3-type and cbb_3-type oxidases indicates there must be significant differences in the local molecular environment of the heme–copper center, and this conclusion is supported by the lack of sequence similarity in these two proteins, even though both contain a similar heme–copper binuclear center where CO or O_2 can bind to either heme or copper.

Like the cytochrome oxidases in *R. sphaeroides*, Nor contains a binuclear center and it has been shown that NorB, the subunit containing the binuclear center, is structurally similar to subunit I from the heme–copper cytochrome oxidases [10]. However, FT-IR indicates that the local molecular environment of the binuclear center in Nor is significantly different from that of either oxidase. In particular, the Fe–CO of Nor from membranes of *R. sphaeroides* is distinguished by its broadness. The broadness of the Nor Fe–CO peak suggests the CO is not constrained and that the local molecular environment surrounding the bound CO is more flexible and polar than in the heme–copper oxidases [21]. Given this, it may therefore be instructive to consider the possible effects of certain structural differences which may affect the binuclear center pocket. NorB from *R. sphaeroides* contains glutamate at residues 190 and 194 in helix VI that correspond to Tyr288 and Leu292 in the subunit I from the aa_3-type cytochrome oxidase of *R. sphaeroides* (data not shown). Residues 190 and 194 are one or two helical turns beyond the likely Fe-ligand His186 which is equivalent to Cu_B ligand His284 in subunit I. Tyr288 in the cytochrome *c* oxidase has been found as a covalent condensation product with the ε–N of His284 in the crystal structures of the cytochrome oxidase from both *P. denitrificans* and beef heart [22,23]. While glutamate in the corresponding position in Nor could not form a similar condensation product, it could readily form a salt bridge with His186, or with a small conformational change be available as a ligand to a non-heme iron. Alterna-

tively, Glu190 and Glu194 might both serve as ligands to non-heme iron in place of His186. In any case, replacement of non-polar groups with glutamate at positions 190 and 194 is expected to greatly increase the hydration in this region of helix VI, and therefore its local conformational flexibility. Exchanging water of hydration at the binuclear center is consistent with the broad CO vibrational transitions observed by FT-IR (this work) and resonance Raman [20]. A third glutamate, Glu259, in NorB has replaced the non-polar Ile355 in helix VII of cytochrome oxidase and is expected to further contribute to a more open, flexible and hydrated Nor structure at the binuclear site. It may be of interest to note that the unrelated Nor from *Fusarium oxysporum* also exhibits an open, hydrated NO binding site [24], suggesting that a flexible, hydrated binding site may be required for NO reduction.

Acknowledgements

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