Fourier Transform Infrared Evidence for Connectivity between Cu_B and Glutamic Acid 286 in Cytochrome bo_3 from *Escherichia coli*†

Anne Puustinen,‡,§ James A. Bailey,‡ R. Brian Dyer,‡ Sandra L. Mecklenburg,‡ Mårten Wikström,*,§ and William H. Woodruff*,‡

Bioscience and Biotechnology Group (CST-4), Chemical Sciences and Technology Division, Los Alamos National Laboratory, Los Alamos, New Mexico 87545, and Helsinki Bioenergetics Group, Department of Medical Chemistry, Institute of Biomedical Sciences and Biocentrum Helsinki, P.B. 8, University of Helsinki, FI-00014 Helsinki, Finland

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ABSTRACT: Photodissociation of fully reduced, carbonmonoxy cytochrome bo_3 causes ultrafast transfer of carbon monoxide (C≡O) from heme iron to Cu_B in the binuclear site. At low temperatures, the C≡O remains bound to Cu_B for extended times. Here, we show that the binding of C≡O to Cu_B perturbs the IR stretch of an un-ionized carboxylic acid residue, which is identified as Glu286 by mutation to Asp or to Cys. Before photodissociation, the carbonyl (C═O)-stretching frequency of this carboxylic acid residue is 1726 cm^{-1} for Glu286 and 1759 cm^{-1} for Glu286Asp. These frequencies are definitive evidence for un-ionized R-COOH and suggest that the carboxylic acids are hydrogen-bonded, though more extensively in Glu286. In Glu286Cys, this IR feature is lost altogether. We ascribe the frequency shifts in the C═O IR absorptions to the effects of binding photodissociated C≡O to Cu_B, which are relayed to the 286 locus. Conversely, the 2065 cm^{-1} C≡O stretch of Cu_B−CO is markedly affected by both mutations. These effects are ascribed to changes in the Lewis acidity of Cu_B, or to displacement of a Cu_B histidine ligand by C≡O. C≡O binding to Cu_B also induces a downshift of an IR band which can be attributed to an aromatic C−H stretch, possibly of histidine imidazole, at about 3140 cm^{-1}. The results suggest an easily polarizable, through-bond connectivity between one of the histidine Cu_B ligands and the carboxylic group of Glu286. A chain of bound water molecules may provide such a connection, which is of interest in the context of the proton pump mechanism of the heme-copper oxidases.

Cytochrome bo_3 from *Escherichia coli* is a member of the large family of structurally related respiratory heme-copper oxidases (Saraste, 1990; Babcock & Wikström, 1992). It is a hydroquinone oxidase, but many of its functional features have proved to be analogous to those of the cytochrome *c* oxidases. In particular, cytochrome bo_3 has been shown to function as a proton pump (Puustinen et al., 1989) and to involve intermediates in the reaction with dioxygen that are analogous to those described for the cytochrome *c* oxidases (Morgan et al., 1995).

Photolysis of carbon monoxide (C≡O) bound to ferrous heme o_3 in the reduced cytochrome bo_3 has been shown to lead to fast transfer of C≡O for binding the Cu_B nearby in the binuclear oxygen-binding site (Dyer et al., 1994, unpublished results), in much the same way as this was shown to occur in cytochrome aa_3 (Alben et al., 1981; Woodruff, 1993; Park et al., 1996). At low temperatures, the C≡O remains bound to the copper for extended time periods, giving rise to a characteristic FTIR[1] Cu_B−C≡O stretch absorbance near 2065 cm^{-1}. Difference FTIR spectra of this photolysis event also show a trough near 1960 cm^{-1} due to the loss of C≡O bound to heme iron. This low-temperature C≡O photolysis procedure has recently been used extensively as a sensitive probe of the effects of site-directed mutations on the binuclear site [cf. Hosler et al. (1993)].

Glutamic acid 286 is one of the best conserved residues among the heme-copper oxidases. It was early on predicted to reside within the membrane, relatively close to the binuclear heme-copper site [see Saraste (1990) and Hosler et al. (1993)]. The recent crystal structures of two cytochrome *c* oxidases (Iwata et al., 1995; Tsukihara et al., 1996) have shown this to be the case (Figure 1).

An early multicopy plasmid strain of the Glu286Gln mutant in cytochrome bo_3 suggested that it was partially or fully active, while Glu286Ala was inactive (Puustinen et al., 1992; Hosler et al., 1993; Thomas et al., 1993a). Other single-copy plasmid strains of Glu286Gln (Mogi et al., 1994) and the analogous mutants of *Rhodobacter sphaeroides* (Hosler et al., 1993) were inactive, however. Recently, this discrepancy was resolved because one of us (A. Puustinen, unpublished results) found that the early Glu286Gln mutant strain of cytochrome bo_3 had reverted to the wild type and that a newly made multicopy Glu286Gln mutant strain of cytochrome bo_3 had less than 5% of the activity of the wild type enzyme. The reverted multicopy plasmid expresses large amounts of cytochrome oo_3 type enzyme (Puustinen et al., 1992), which was useful in studies of intramolecular electron transfer (Morgan et al., 1993), but this must be ascribed to the plasmid expression and not to mutation of Glu286.

Svensson-Ek et al. (1996) showed that the Glu286Ala mutation blocks the oxygen reaction of cytochrome bo_3,

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‡ Los Alamos National Laboratory.

§ University of Helsinki.

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[1] Abbreviations: FTIR, Fourier transform infrared; wt, wild type.

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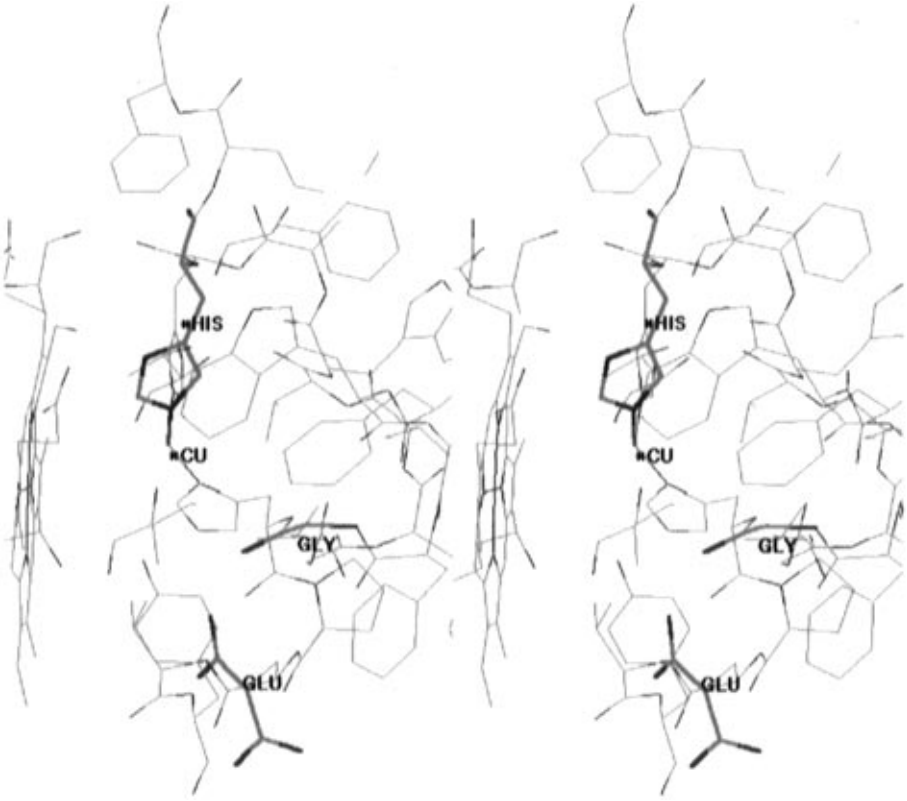

FIGURE 1: Binuclear heme-copper site. Stereoview based on the crystal structure of cytochrome aa_3 from bovine heart mitochondria (Tsukihara et al., 1996). Residues His334, Gly283, and Glu286 and Cu_B are highlighted (*E. coli* cytochrome bo_3 numbering; these residues are His291, Gly239, and Glu242 in the mitochondrial enzyme). Two orientations of the side chain of Glu286 are shown simultaneously; in the crystal structure, the carboxyl oxygens are directed downward. An alternative stable orientation may be reached by rotation around the Cβ−Cγ bond so that the carboxyl oxygens point upward, toward His334. This conformation may be stabilized by hydrogen bonding to the backbone oxygen of Gly283 (see the text).

simultaneously blocking the uptake of protons. Ädelroth et al. (1997) recently reported similar results for the Glu286Gln mutant of cytochrome *c* oxidase from *Rh. sphaeroides*. These findings suggest that Glu286 plays an important role in the catalytic mechanism. However, an assessment of whether the carboxylic residue is directly involved in proton translocation has been difficult due to the enzymatic inactivity of mutants tested thus far at this locus. One of us (A. Puustinen) recently found that the enzymatic activity of the Glu286Cys mutant of cytochrome bo_3 is sufficient for turnover experiments. Such experiments in whole *E. coli* cells and proteoliposomes have now shown that proton translocation is abolished in the Glu286Cys mutant but that this activity is retained in Glu286Asp (Verkhovskaya et al., 1997). The phenotype of Glu286Cys thus resembles the Asp135Asn mutant (Thomas et al., 1993a), as well as the result of changing two asparagines near the Asp135 residue (Garcia-Horsman et al., 1995). From all this information, it seems clear that Glu286 is a key residue for the proton-translocating mechanism.

Here, we show by FTIR experiments that photoinduced CO binding to Cu_B perturbs a carboxylic acid IR stretch, which we identify as being due to Glu286. Conversely, mutations at the Glu286 locus change the CO stretch of the Cu_B−CO compound. We suggest that these results may be ascribed to connectivity between a histidine ligand of Cu_B and the carboxylic group of Glu286, which may be provided by water molecules bound to this domain in the enzyme. Such a connection might be of key importance for the mechanism of proton pumping (Morgan et al., 1994).

MATERIALS AND METHODS

Cytochrome bo_3 Mutants. The mutants were constructed according to previously published methods (Lemieux et al., 1992; Thomas et al., 1993b) and confirmed by DNA sequencing, and also by DNA sequencing of double-stranded expressing plasmid from the host strain. Glu286Asp is in the pJT40 plasmid and is expressed in the host strain GO105 (*cyo* Δ*cyd rec*A; Calhoun et al., 1993). Glu286Cys is in the plasmid pHTC8, a derivative of pL1 (Lemieux et al., 1992), from which all cysteines from subunit I (Cys136Leu, Cys322Ser, Cys432Leu, and Cys512Leu), subunit III (Cys37Val, Cys136Ala, and Cys150Leu), and subunit IV (Cys64Ala) were mutated. These cysteines are not conserved, and were mutated to residues commonly found in the respective locus among the heme-copper oxidases. The phenotype of the cysteine-free pHTC8 expressed in GO105 cells exhibits turnover and proton-translocating activities similar to those of the wild type (A. Puustinen, unpublished). The Glu286Cys mutant plasmid was transformed into the host strain GL101 (*cyo sdh rec*A) (Lemieux et al., 1992), because it does not complement aerobic growth of *E. coli* well enough. All used plasmids contained a construct for a "histidine tag" to facilitate isolation of the enzyme (Rumbley et al., 1997).

FTIR Spectroscopy. Bacterial growth conditions and purification of cytochrome bo_3 enzymes were as described by Morgan et al. (1995). Enzyme samples for FTIR spectroscopy were diluted 10-fold into 0.1 M sodium phosphate and 0.01% (w/v) *n*-dodecyl β-maltoside (Anatrace) at pH 7.4 in heavy water (D_2O) and concentrated again. This

was repeated once. Anaerobic enzyme (0.3−0.4 mM cytochrome bo_3) was reduced with dithionite under an atmosphere of argon, after which 1 atm of CO was flushed over the reduced sample. The sealed sample was mixed a few times to facilitate CO equilibration with the solution. After 30 min of incubation with CO, the sample was mounted between CaF_2 windows with 25 μm Teflon spacers. A Perkin-Elmer 1760X FTIR spectrometer equipped with an APD Cryogenics cryostat and a mercury cadmium telluride (MCT) detector was used to record the FTIR spectra at 80 K. The reference spectrum (in single-beam mode, 64 scans) was collected just before photolysis (Nd:YAG laser, 532 nm, two 3 mJ flashes with a 10 ns duration), after which several spectra (64 scans each) were recorded. Difference spectra were obtained by subtraction of the reference spectrum. Samples were warmed to 260 K for 10 min to allow the CO to relax back to the heme iron, and then cooled back to 80 K to repeat the experiment.

Data Analysis. Resolution of overlapping infrared peaks and difference features was performed on a Power Macintosh 9500/150 computer using the program Igor Pro (Wavemetrics, Inc.). Peak shapes were assumed to be Gaussian in all cases. This assumption was verified by fits to isolated peaks, and is reasonable considering the inhomogeneous broadening expected of infrared absorbances in proteins. Fits to the carboxylic acid difference features in the 1700−1800 cm^{-1} region were performed using a two-Gaussian function with the baseline offset equal to a constant. The peak location and height were constrained using values obtained from the frequencies and intensities of the COOH peaks for the Fe−C≡O forms of the Glu286 and Glu286Asp samples in the respective absolute absorbance spectra. The peak widths are expressed as the half-width at 1/e height; initial guesses were 6 cm^{-1} in all cases. The Fe−C≡O and Cu−C≡O peaks were fit to a three-Gaussian function plus baseline offset. In all cases, the iterative fitting routine converged promptly without constraints; the "best-fit" criterion was minimum χ^2. The experimental errors for the fitted frequencies and amplitudes were estimated as ± 0.5 cm^{-1} and $\pm 5 \times 10^{-5}$ optical density (OD) units, respectively.

RESULTS AND DISCUSSION

Figure 2 shows the FTIR difference spectrum obtained upon C≡O photolysis (light minus dark) from reduced CO-treated cytochrome bo_3 (wt, wild type enzyme). The well-known C≡O stretching features are seen at 1960 cm^{-1} (negative) and 2065 cm^{-1} (positive) due to binding of the C≡O to heme iron before and to Cu_B after photolysis, respectively. In addition, there are changes in the amide I region, below 1700 cm^{-1}, which have been ascribed mainly to the C═O stretches of protein backbone carbonyl moieties, and numerous small changes in side chain absorbances.

The difference spectrum also reveals a derivative-shaped feature in the carboxylic acid carbonyl (C═O) stretching region, with a peak at 1731 cm^{-1} accompanied by a trough at 1724 cm^{-1} (Figure 2, inset; Figure 3, trace A). Carboxylic acid features in the 1700−1800 cm^{-1} region are definitive evidence of a protonated R-COOH structure rather than a deprotonated carboxylate. Thus, the group in question must be buried in the protein structure so that it does not sense the ambient pH of 7.4. A Gaussian resolution of the difference feature reveals that it corresponds to an absorbance

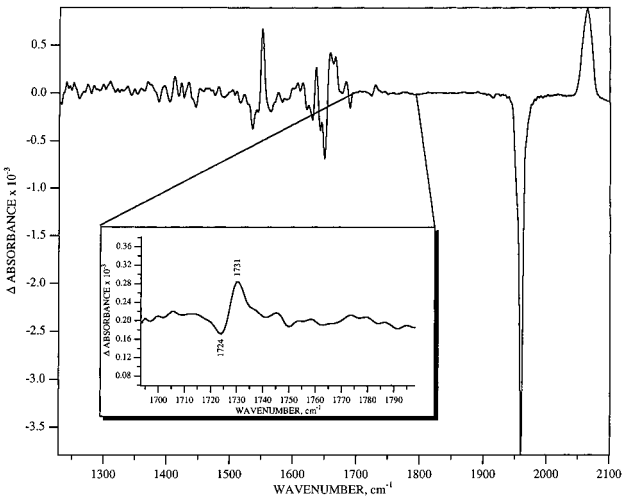

FIGURE 2: FTIR "light" minus "dark" difference spectrum for the CO adduct of wild type cytochrome bo_3. Average of 1536 spectra.

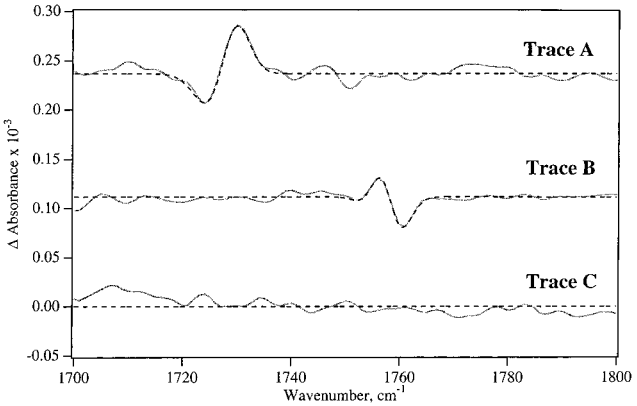

FIGURE 3: Light minus dark FTIR difference spectra in the carboxylic acid C═O stretching region: (A) wild type enzyme (average of 1536 spectra), (B) Glu286Asp mutant (1344 spectra), and (C) Glu266Cys mutant (1644 spectra). In traces A and B, the dashed lines are the fits to the derivative-shaped difference features created by subtraction of the contributing Gaussian fits to the C═O stretching absorbances before and after photodissociation (see Materials and Methods). In trace C, the dashed line is simply a constant baseline. Parameters of the Gaussian fits are as follows (peak height, absorbance units; peak maximum, cm^{-1}; half-width at 1/e height, cm^{-1}): wild type (A) before photodissociation, 8×10^{-5}, 1726.3, 5.1; after photodissociation, 1.2×10^{-4}, 1729.5, 4.2; Glu286Asp mutant (B) before photodissociation, 1.1×10^{-4}, 1758.6, 3.3; after photodissociation, 1.1×10^{-4}, 1757.6, 3.0. Note that the frequency shift for the Glu286Asp mutant is much smaller and in the opposite direction compared with that of the wt enzyme (see the text).

that shifts from 1726.3 cm^{-1} (Fe−C≡O form) to 1729.5 cm^{-1} (Cu_B−C≡O form). Figure 3 (trace B) shows that in the Glu286Asp mutant this carbonyl stretch feature is replaced by a peak and trough at 1756 and 1761 cm^{-1}, respectively, and the Gaussian fit shows the contributing carboxylic acid absorbances to be at 1758.6 cm^{-1} (Fe−C≡O) and 1757.6 cm^{-1} (Cu−C≡O). All of these bands are lacking in the Glu286Cys mutant (Figure 3, trace C). We may thus uniquely assign these IR bands to the carboxylic acid stretches of glutamic acid and aspartic acid, respectively, at the 286 locus.

The C═O stretch of Glu286 at ca. 1730 cm^{-1} in the wild type enzyme is similar in frequency to those of known cyclic carboxylic acid dimers (Dollish et al., 1974), and thus indicative of a strongly hydrogen-bonded carboxylic acid.

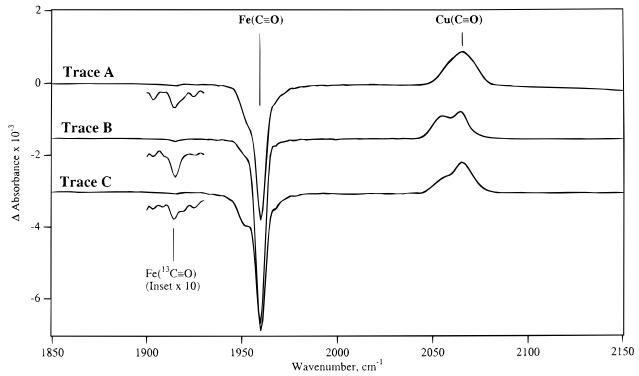

FIGURE 4: Light minus dark FTIR difference spectra in the carbon monoxide C≡O stretching region. (A) wild type enzyme, (B) Glu286Asp mutant, and (C) Glu286Cys mutant. The spectra are scaled to one another so as to make the integrated intensities of the Fe−C≡O peaks equal. Fe−^{13}C≡O satellite peaks are also evident at 1915 cm^{-1}, with 1.1% of the Fe−^{12}C≡O intensity. The Fe−^{12}C≡O and Cu−^{12}C≡O peak envelopes could be fit satisfactorily to three Gaussian contributors (see Materials and Methods).

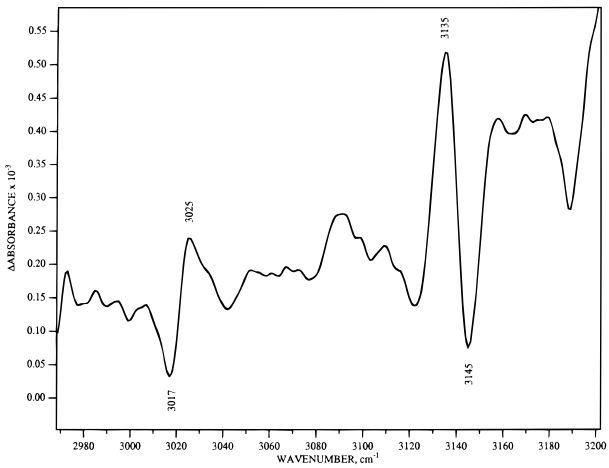

FIGURE 5: Light minus dark FTIR difference spectrum in the 3000−3200 cm^{-1} region. The spectrum is for the wild type cytochrome *bo*$_3$.

In contrast, non-hydrogen-bonded carboxylic acid monomers absorb at a considerably higher frequency (ca. 1790 cm^{-1}). Consequently, in the Glu286Asp mutant with its C=O stretch at ca. 1758 cm^{-1}, we may infer that the side chain, which is one methylene group shorter than that of glutamic acid, is still hydrogen-bonded when positioned at the 286 locus, but less strongly than the glutamic acid. In both the wt and Glu286Asp cases, the peaks are broader when CO is bound to Fe ("dark") than when CO is bound to Cu ("light"), which suggests that the hydrogen-bonded environment of residue 286 is more homogeneous in Cu−CO than in Fe−CO.

Figure 4 shows the FTIR difference spectra in the frequency range of the carbon monoxide C≡O stretching absorbances of both Fe−CO and Cu−CO. It is noteworthy that the C≡O peaks represent one single chemical bond in a protein having a molecular mass of approximately 100 kDa. Moreover, the satellite peak for natural isotopic abundance (1.1%) Fe−^{13}CO is clearly observable at 1915 cm^{-1}, with signal-to-noise of >5. The exquisite sensitivity of FTIR difference spectroscopy to protein structural changes under functional conditions is obvious.

It is clear from Figure 4 that the two mutations in the 286 locus affect the shape and size of the C≡O stretching band of Cu$_B$−CO, and also that of Fe−CO, though less extensively. We conclude not only that the binding of CO to Cu$_B$ perturbs the carboxylic acid IR absorption of Glu286 but also that a mutation at the latter site reciprocally affects the IR absorption of C≡O, when bound to Cu$_B$ and to heme iron, and thus the structural nature of the metal-bound C≡O moiety. However, the effects of mutating Glu286 on Fe−CO and Cu−CO do not correlate with one another; for example, the Fe−CO peak of the Glu286Asp mutant is the most homogeneous of the three, but its Cu−CO peak is the most clearly split. Thus, the structural effects of a mutation at the 286 locus are quantitatively different at the two metals, and appear to affect Cu$_B$ more extensively than the heme iron.

Figure 5 shows the difference spectrum in the 3000−3200 cm^{-1} region. The shift of a peak near 3145 cm^{-1} to ca. 3135 cm^{-1} upon binding of C≡O to Cu$_B$ is evident. This is precisely the frequency region expected for the C−H stretching modes of histidine imidazole (Dollish et al., 1974); other aromatic C−H stretches, including the methine C−H stretches of hemes (Alben, 1978), are expected to be lower in frequency, and stretches such as O−H and N−H are considerably higher. While we cannot definitively assign this feature without isotope data, we tentatively attribute the observed frequency shift to weakening of a C−H stretching mode of a histidine imidazole group, as a consequence of C≡O binding to Cu$_B$. Figure 5 also shows shifts near 3020 and 3080 cm^{-1}, which may arise from a C−H stretch of a heme vinyl or methine bridge. None of these shifts are affected significantly by the mutations at the 286 locus.

These results provide a definite identification of the carboxylic acid IR feature of Glu286. The low frequency indicates that the side chain is effectively hydrogen-bonded, which is of particular interest because the crystal structures show no obvious hydrogen-bonding partners. The extent of this hydrogen bonding decreases, and the frequency shift of the carboxylic acid carbonyl group becomes smaller in magnitude and opposite in sign, upon replacing the glutamic with aspartic acid. It is especially noteworthy that the carboxylic acid absorption frequency of Glu286 depends on whether C≡O is bound to heme iron or to Cu$_B$, and conversely, that the C≡O stretch of Cu$_B$−C≡O depends on the nature of the side chain at the 286 locus. We suggest that this may be due to a through-bond connectivity between a Cu$_B$ histidine ligand and the carboxylic group. We envision two ways in which this might occur. First, the binding of CO to Cu$_B$(I) tends to make the copper a stronger Lewis acid due to the π bonding between CO and Cu$_B$. As a result of this, the bonds between Cu$_B$ and its histidine imidazole ligands strengthen, and this effect is relayed to the carboxylic group. Alternatively, the binding of CO to Cu$_B$ may cause one of the histidine ligands to be displaced from the copper, as has been suggested previously (Woodruff et al., 1991). This clearly will alter the basicity of the unbound histidine imidazole and, to a lesser extent, that of the imidazoles still bound to the copper, as well as the Lewis acidity of Cu$_B$ itself. As in the first case, these effects are relayed to the Glu286 side chain.

We must first consider whether such an effect could take place between Glu286 and the Cu$_B$ ligand His284 via an inductive effect through the covalent bonds of the protein backbone between these two residues, which are separated by Pro285 (Figure 1). This pathway has 11 bonds (excluding the imidazole ring and the carboxylic group itself), only two

of which have π character (the amide bonds). Hence, there is no conjugation along this pathway. It seems unlikely that a through-bond effect could conduct the inductive effect suggested here over the required distance, and engender in the 3 cm^{-1} upshift in $\nu_{(C=O)}$ of Glu286. However, the $\approx$1 cm^{-1} downshift observed for Glu286Asp might be the result of such a weak effect (but see below).

Since the crystal structures at 2.8 Å do not reveal any other obvious connectivity (Iwata et al., 1995; Tsukihara et al., 1996), we suggest that one of the Cu_B ligands (most likely His334 since it is closest to Glu286; Figure 1) may be linked to the carboxylic acid residue through bound water molecules. For this to be effective, the Glu286 side chain might have an alternative orientation in which the carboxylic group points toward Cu_B, where it may be stabilized by hydrogen bonding to the nearby backbone oxygen of Gly283 (Figure 1). In any case, a hydrogen-bonded water array would satisfy the requirement of a highly polarizable pathway needed to transmit the inductive or ligation/deligation effects at Cu_B over relatively long distances, and would also be consistent with the effective hydrogen bonding of the carboxylic acid.

The smaller amplitude of the Glu286Asp shift in the difference spectrum, relative to that of Glu286, is shown by the Gaussian resolutions to be due to the smaller frequency shift (1.0 ± 0.5 cm^{-1} for Glu286Asp compared to 3.2 ± 0.5 cm^{-1} for Glu286) and not to the amplitude of the absorbances which contribute to the difference features. The shift may be smaller simply because the shorter aspartic acid side chain may be less effectively hydrogen-bonded to the network leading to the copper ligand. For this reason, the through-hydrogen-bond effects may be weaker. Thus, if a hydrogen-bonded water pathway were the conductive element between the histidine ligand of Cu_B and the carboxylic acid in both Glu286 and Asp286, the different sign of the absorption shift might be caused by different linkage of the water chain to the carboxyl group in the two cases. H bonding to the C−OH and C═O groups of the carboxyl, respectively, is expected to result in shifts of opposite sign, everything else being equal. In this case, the carboxylic acid configurations of the glutamic and aspartic residues in the 286 locus might differ by a 180° rotation around the terminal C−C bond.

Recently, Hellwig et al. (1996) reported a shift to a lower frequency of a carboxylic acid IR stretch upon reduction of cytochrome aa_3 from *Paracoccus denitrificans*. Lübben and Gerwert (1996) reported very similar data for reduction of both cytochrome aa_3 from *Rh. sphaeroides* and cytochrome bo_3 from *E. coli*. In both these studies, the bands of the oxidized and reduced enzyme (in D_2O) appeared at ca. 1741 and 1727−1729 cm^{-1}, respectively, in the difference spectra. From our study, it seems likely that these workers were also observing a frequency shift of the carboxylic stretch of Glu286. We suggest that the redox-dependent shift may be explained in the same way as we interpret the present C≡O binding data, without evoking protonation/deprotonation of the carboxyl group or involvement of two carboxyl groups. Reduction of Cu_B makes the copper a poorer Lewis acid so that the resulting inductive effect on the carboxylic residue should be manifested in the direction opposite from the effect of binding C≡O to Cu_B, which was indeed observed (Hellwig et al., 1996; Lübben & Gerwert, 1996). The fact that the IR band of the reduced enzyme appeared near 1727−1729 cm^{-1} in the difference spectrum while the band of the C≡O-reduced enzyme appears near 1724 cm^{-1} (Figures 2 and 3) could be due to the influence of C≡O bound to heme iron. This is consistent with our finding that the Fe-bound C≡O stretching feature is sensitive (though only slightly) to mutations at the 286 locus (Figure 4).

We think that it is unlikely that the redox-linked effect on the carboxylic absorption band is due to proton transfer (Hellwig et al., 1996; Lübben & Gerwert, 1996), because proton uptake coupled to reduction of the enzyme has been ascribed to a proton-conduction pathway involving the Lys362 residue in helix VI of subunit I (Siletsky et al., 1996), which is distinct from the pathway thought to involve the residues Asp135 and Glu286 (Iwata et al., 1995). The latter pathway is, instead, likely to be engaged in proton uptake coupled to the phase of the catalytic cycle that carries out the oxygen chemistry [cf. Babcock and Wikström (1992), Thomas et al. (1993a), Garcìa-Horsman et al. (1995), and Siletsky et al. (1996)].

The present results infer a hydrogen-bonded connectivity between Glu286 and a histidine copper ligand, possibly through bound water molecules. This is of obvious interest in view of the "histidine cycle" model of proton translocation by the heme-copper oxidases (Morgan et al., 1994). It may provide a key protonic connection, also proposed by Iwata et al. (1995), of this mechanism, which is "missing" from the 2.8 Å crystal structure. If so, it implicates His334 in this mechanism, rather than His333 (Iwata et al., 1995) or His284 ligands (Morgan et al., 1994) as previously suggested.

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