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Sum Frequency Generation Studies of Membrane Transport Phenomena

R. Brian Dyer* and Andrew P. Shreve

Abstract

This is the final report of a three-year, Laboratory Directed Research and Development (LDRD) project at the Los Alamos National Laboratory (LANL). The objective of this work is to study the transport of protons and ions across biological membranes, one of the most fundamental processes in living organisms, critical for energy transduction in respiration and photosynthesis and for a wide variety of cellular signal transduction events. Membrane protein structure and function, in particular proton and ion pumping are poorly understood. We have developed sum frequency generation (SFG) spectroscopy for the study of membrane phenomena, a nonlinear spectroscopic technique that is uniquely sensitive to interfaces and with demonstrated structural specificity. We have used SFG and conventional vibrational spectroscopic approaches to study proton transport processes in cytochrome c oxidase. A key finding has been the identification of vibrational modes associated with proton labile groups, including a glutamic acid near the redox active binuclear center and structural waters. These groups are sensitive to the ligation and redox states of the metal centers and hence are ideal candidates for coupling redox energy to proton transport processes.

Background and Research Objectives

One of the most fundamental processes in living organisms is the movement of protons and other ions across biological membranes. This process, which is critical for energy transduction in respiration and photosynthesis and for a wide variety of cellular signal transduction events, is generally carried out by membrane-bound proteins. Because of the difficulty of obtaining structural and dynamic information about both membrane-bound proteins and membranes, a molecular-level understanding of proton and ion pumping has remained elusive. We propose to apply newly emerging spectroscopic techniques that yield surface-specific structural and dynamic information to several different problems in the transport of ions across membranes.

Processes involving membranes and membrane proteins are difficult to study for several reasons. Structural information is hard to obtain because membrane proteins are

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often complex and always difficult to crystallize. Even if a high-resolution structure of an isolated membrane protein can be determined, questions will remain about how interactions with the membrane and solvent influence the structure of the protein in its native environment. Further, even if all the desired structural information were known, the functional dynamics of the protein in its membrane environment still need to be elucidated before the function of the protein is understood at a molecular level. For example, ten years have passed since the determination of the structure of photosynthetic reaction centers, and as yet a full understanding of the efficiency and mechanisms of photosynthetic electron- and proton-transfer reactions does not exist. In general, for membrane proteins, especially those functioning in ion transport, functional dynamics can also involve nearby water, ions, or even the membrane itself, and the study of these is extremely difficult because of sample heterogeneity and large background signals from the bulk solution.

These difficulties, combined with the importance of membranes and membrane proteins, provide an excellent opportunity for the development of new techniques. An ideal method for studying membranes would be one sensitive only to the surface region, in order to discriminate against the bulk background, and one that yields both structural and kinetic information. The second-order nonlinear optical technique of sum-frequency generation (SFG) satisfies all of these criteria [1-3]. The experiment involves illuminating a sample with light of two wavelengths. The second-order nonlinear response of the sample then generates light at a third wavelength corresponding to the sum of the two input frequencies. Unlike the linear response, which leads to the familiar optical effects of absorption or refraction but does not generate new wavelengths, the second-order response is zero by symmetry for any isotropic portion of the sample. Thus, the intensity of the sum-frequency light is sensitive to ordered molecular structures and anisotropic local electric fields at surfaces, but there is no contribution from the isotropic solution.

In vibrational SFG, one of the two incident wavelengths is in the vibrational infrared and by monitoring enhancement of the sum-frequency intensity as the infrared wavelength is tuned into resonance with a molecular infrared (IR) absorption, the vibrational spectra of molecules in the surface region can be determined with no interference from the much more concentrated bulk solution. In addition to being surface specific, SFG can be used as a time-resolved spectroscopy [3], and thus can provide time-resolved vibrational spectra of molecules in the surface region. The advantages of time-resolved vibrational measurements as structure-specific probes of protein reactivity and dynamics are well established [4]. The structural specificity results because specific protein structures determine the frequency, intensity and line-widths of vibrational absorptions. For example, the amide vibrations are sensitive to secondary structure. Higher-order

structure is sometimes difficult to study by traditional infrared spectroscopy, but SFG can probe the orientation of secondary structural elements with respect to the symmetry-breaking membrane surface and will be an excellent new probe for changes in tertiary structure. Both SFG and traditional IR techniques can follow dynamics on time scales ranging from a single vibrational period (10 to 100 fs) to hours, and the sensitivity of transient IR is sufficient to detect changes in the IR spectrum of a single amino acid residue in a medium sized protein, while SFG has been demonstrated to be sensitive to fractions of a monolayer of adsorbed molecules.

Both SFG and the related technique of second-harmonic generation (SHG), which involves using one visible input wavelength and detecting signal at half that wavelength (the second-harmonic frequency), are becoming widely used techniques in chemistry and physics [1-3,5]. For example, SFG has been used to study the structure of water molecules at the interface between water and air, or between water and quartz [2]. These studies were able to measure the orientation of water molecules at the surface, determine their vibrational spectrum, and probe the extent of disruption of hydrogen bonding in the surface layer of molecules, all with no interference from the bulk water. Yet, surprisingly, given the information that can be obtained about structures, orientational ordering and dynamics at interfaces, application of these techniques to the study of biological membranes has been extremely limited. There have been a few SHG experiments on bacteriorhodopsin [6], but despite these demonstrations of feasibility, systematic SHG studies of membranes or membrane-bound proteins have not been done and we are aware of no applications of the more informative SFG technique in biological systems.

The first scientific problem we addressed in this project was to examine the structure and function of the terminal oxidases of respiration using IR and SFG techniques. The heme-copper oxidases act as the terminal oxidase systems of cellular respiration in all plants and animals, and many simpler organisms. As such, this enzyme "superfamily" is the keystone of aerobic life and respiratory bioenergetics. It is responsible for perhaps 90% of the biological O_2 reduction on earth, and catalyzes the reactions which yield nearly half of the total energy of cellular respiration. Two major types of heme-copper oxidases are known at present, based on the reducing substrate: the cytochrome *c* oxidases (CcO), which accept electrons from cytochrome *c*, and the quinol oxidases which accept electrons from quinol. Both types catalyze the four-electron reduction of O_2 by the reducing substrate at turnover rates as fast as 1000 s^{-1} . They conserve the energy of this reaction by creating a transmembrane proton gradient by two mechanisms: "scalar" uptake of protons from one side of the membrane in the course of the reduction of O_2 to H_2O ,



and "vector" translocation of an additional four protons per mole of O_2 from one side of the membrane to the other, via a "redox-linked proton pumping" process. The mechanism by which redox-linked proton pumping occurs is unknown, and is one of the major issues in modern bioenergetics.

The energy conversion mechanisms of the heme-copper oxidases can be separated into a few basic processes including: the behavior of the exogenous ligand (e.g. O_2), its entrance into the protein, coordination, and activation; the behavior of the metal centers as they perform their coordination, activation, and electron transfer functions; the electron transfer reactions and the phenomena that determine their rates; the operation of the proton pump that displaces the "vector protons" against the transmembrane pH gradient; the coupling of proton pumping to the electron transfer free energy; the delivery of the "scalar protons", which combine with O_2 to form H_2O , to the active site; and the structural responses of the polypeptide itself, and its sidechains, to the foregoing processes.

Vibrational spectroscopies are sensitive to all of these effects. We have demonstrated our ability to follow with structural specificity the dynamics of ligation, electron transfer, and polypeptide response over many orders of magnitude of time (subpicosecond to static). The dynamics of the proton-motive structures and dynamics of the oxidases are observable in the same manner. This project developed the capability to monitor changes in the time-resolved IR and SFG signals and match these with the kinetics of proton uptake and release or the protonation and deprotonation of specific residues. Since the IR and SFG signals are quite sensitive to changes in tertiary structure, we will then be able to test models of the proton-pumping mechanism that suggest structural changes during the redox cycle that expose specific residues to the solvent on either the cytoplasmic or extracellular side of the membrane. Protonation and deprotonation of these residues is then supposed to account for proton uptake and release, and in addition, these protonation and deprotonation steps may drive subsequent structural changes of the protein.

The long-term goal of our studies is to understand, at a molecular level, the structure and dynamics of the proteins, membranes and surrounding solvent that facilitate and control the functions of membrane-bound proton and ion pumps. Our studies concentrated on the heme copper oxidases from bacteria and higher organisms. We used both IR and SFG spectroscopies to probe the structure and dynamics of the active sites of these proteins. The latter nonlinear optical technique is sensitive to interfacial regions in a complex solution, and can provide both the structural information of vibrational

spectroscopy and the dynamic information of time-resolved spectroscopy, making it an ideal approach for the study of membrane phenomena.

In addition to these scientific goals, which will also serve to pioneer the application of SFG spectroscopy for the study of biological interfacial phenomena, this project developed two important technical advances in the SFG technique itself. These are multichannel detection of the SFG signal and the determination of mid-vibrational IR spectra using SFG.

Importance to LANL's Science and Technology Base and National R&D Needs

Scientifically, we seek to develop an understanding of molecular-level mechanisms for vectorial proton and ion transport across membranes. To do this, new approaches need to be developed to allow dynamic study of structures important for membrane protein function. To this end we are developing SFG, a nonlinear, time-resolved, surface-specific spectroscopy, capable of identifying and studying molecular-level structures on time scales from femtoseconds to kiloseconds. Membranes play a vital role in living organisms. The bioenergetics of all higher organisms, for example, depend on membranes to bind energy-transducing proteins and to form a barrier across which vectorial proton transport can store energy. Photosynthetic proteins, including bacteriorhodopsin, couple photochemistry to proton pumping, while respiratory energy-transduction proteins use chemical redox energy to pump protons. In both cases the proton gradient is used by a separate enzyme system to power the synthesis of ATP, the ubiquitous energy currency of the cell.

In addition to proton pumps, a number of proteins transport other ions across membranes, often as part of signal-transducing processes. The resulting trans-membrane concentration differences, and the corresponding trans-membrane electrical potential, must be maintained for any cell to function properly. SFG offers a new and exciting way to study, in real time, the vectorial transport of ions across membranes. The methods developed here can also be applied to other biological transport systems such as bacteriorhodopsin and ion channels such as colicin. Because SFG is sensitive to processes at membrane surfaces, including the changing of trans-membrane charge distributions, and because it can yield structural information, we expect that it will be a valuable new method for the study of structural control of ion channel function. The present work provides much needed experimental data for testing proposed mechanisms of proton pumping functions.

Technically, this project pioneered the application of the SFG technique in biological systems. Based on the emerging use of this technique in chemistry and physics, we anticipate that it will become a valuable and much-used technique in the study of biological interfaces. In addition, we have made two very important advances in the SFG

technique itself. First, we have demonstrated that an SFG spectrum can be obtained using spectrally broad infrared light, with the resulting sum-frequency signal spectrally dispersed onto a multichannel detector. This introduces a multichannel advantage into the SFG experiment, and substantially increases the signal-to-noise ratio of a spectrum obtained within a given total signal accumulation time. Also, because of existing technology in our laboratory for generating intense infrared light in the mid-frequency vibrational range, we are in a unique position to extend spectral studies with SFG into this range. Both of these technical advances will be important for general studies of molecular structure at surfaces, whether in chemical, physical or biological systems.

Finally, the information that results from these studies about the mechanisms of proton- and ion- transport processes should suggest strategies for reproducing these mechanisms in synthetic materials. If this can be accomplished, non-biological systems may be designed which perform functions analogous to the important energy- and signal-transduction processes that occur at and across biological membranes. Once the basic principles of designing such materials are understood, the entire range of modern, sophisticated synthetic techniques can be brought to bear to design molecular level biomimetic devices, including medically important biosensors.

Scientific Approach and Accomplishments

Our general experimental approach involves both static and time-resolved SFG (and IR) spectroscopy. There are several convenient vibrational absorptions that can be used to monitor changes in protein structure. A number of amide modes are associated with peptide bonds and these bands can often be correlated with specific secondary structures, so changes in the positions and/or intensities of these bands are diagnostic of structural changes. For example, the amide I (Raman and IR active), amide II (IR active) and the amide III (Raman active) regions of the vibrational spectra of proteins are sensitive to both the conformation of the polypeptide backbone and to hydrogen bonding patterns between the backbone C=O and N-H moieties. The exact pattern of these bands in the protein spectrum has been extensively analyzed in terms of secondary structure. The frequency of the backbone N-H stretch motion, band A (Raman and IR active), is also known to be directly correlated to the strength of hydrogen bonding. Thus, for example, it is possible to monitor changes in hydrogen bonding that might be associated with conformational changes.

In certain situations, changes in side chain vibrations can also be identified in spectra of large proteins. Specific targets are residues that have ionizable side chains, including the carboxylic acids thought to play a role in proton pumping in oxidases. The

carboxylic acid group has an intense IR absorption in the 1690 - 1760 cm^{-1} spectral region that shifts down by over 100 cm^{-1} upon deprotonation. Also, since SFG is sensitive to the ordering of molecules with respect to the surface that breaks the isotropic symmetry of the system, this technique can be used to probe tertiary structure in membrane proteins. For example, one would be able to follow changes in the orientation of an α -helix with respect to a membrane surface using SFG. We also note that any mode that is both Raman and IR active is expected to be SFG active, so the SFG experiments will be able to examine the amide I and the band A spectral regions, which are the most informative for structural determination.

The samples are either suspensions of protein-containing membrane particles or proteins reconstituted into a planar lipid bilayer. Techniques for the preparation of either are completely standard. Static and transient IR were performed using standard techniques (many of which were developed by the principal investigator). SFG experiments are a straightforward extension of time-resolved IR techniques. In fact, the most difficult aspect of an SFG experiment is generation of the intense IR light, which is already routinely done in our laboratory.

Progress on this project has centered on technical development of the SFG methods. This work has focused on developing the laser technology required to carry out the SFG experiments, and on developing the detection methods necessary for the detection of the SFG signals. The initial step was to bring into operation a high-repetition-rate, high-power femtosecond laser. This system is based on a titanium:sapphire oscillator and regenerative amplifier, and generates high power pulses of approximately 100 fs duration at 800 nm. To generate radiation in the vibrational infrared spectral region, as required for SFG, an optical parametric generator/amplifier (OPA) has been built. The OPA uses β -barium borate nonlinear optical crystals, and when pumped at 800 nm, generates high-power pulses in the near infrared with wavelengths tunable from approximately 1100 nm to 2200 nm. In fact, the output of the OPA consists of pulses at two wavelengths, with the shorter wavelength pulse referred to as the signal and the longer wavelength referred to as the idler. By adjusting the wavelengths of the signal and idler, a configuration can be achieved where the difference frequency between the signal and idler corresponds to a specific wavelength in the vibrational infrared. Thus, after the OPA, the signal and idler pulses are passed through another nonlinear optical crystal, silver gallium sulfide, to generate radiation at the wavelength corresponding to the difference frequency of the signal and idler.

The construction of all of these nonlinear optical devices has been completed. In the current configuration, infrared light has been generated at wavelengths ranging from 3 to 10 μm ; however, tunability out to 12 μm can be easily achieved with the same apparatus. At present we have been able to demonstrate the generation of tunable infrared pulses of approximately 1 μJ of energy. In addition to the laser system, a sensitive detection system is also required for the SFG experiments. To that end, we have constructed a sensitive photon-counting apparatus that will allow us to detect signals that may average well less than one photon per incident laser pulse. The apparatus uses a very low dark noise photon counting photo-multiplier tube and a gated photon counter.

We have developed methods of making phospholipid vesicles and reconstructing membrane proteins such as the oxidases into vesicles. The permeabilities of the vesicles in the presence and absence of the reconstituted proteins were also measured. Phospholipid vesicles were prepared by a cholate dialysis method with and without protein. The enzyme-reconstituted vesicles were prepared by combining the specific enzyme, pretreated with cholate, with phospholipids that had been sonicated in 2% cholate. This was followed by dialysis for 48 hours against three to four changes of HEPES buffer. The resulting reconstituted vesicles were tested for protein incorporation and membrane efficacy. Including a pH-sensitive dye in the reaction mixture during sonication and dialysis allowed incorporation of the dye into the reconstituted vesicles. Permeability was measured by creating a pH gradient across the membrane and following the redistribution of protons across the membrane by measuring the fluorescence of the incorporated pH sensitive dye. Permeabilities were found to be the same in the absence and presence of protein, indicating that the reconstitution of enzyme into phospholipid vesicles does not cause significant leaks in the vesicles, an important result for future proton pumping measurements.

In cell respiration, energy for the synthesis of ATP is conserved primarily by proton translocation, creating an electrochemical proton gradient across the mitochondrial or bacterial membrane. Here we address the molecular mechanism by which the heme-copper oxidases translocate protons. Reduction of O_2 to water takes place at a heme iron-copper (Cu_B) center, and protons enter from one side of the membrane through a 'channel' structure in the enzyme (see CcO structure in Figure 1). Statistical-mechanical calculations predict bound water molecules within this channel. Hydrogen-bonded water molecules connect the channel further via a conserved glutamic acid residue to a histidine ligand of Cu_B , which may define the proton-translocating pathway. Perturbing the Cu_B ligand structure shifts an infrared mode that may be ascribed to the O-H stretch of bound water. This is sensitive to mutations of the glutamic acid, supporting connectivity to the histidine. The glutamic acid side chain may have to move during proton transfer because proton

translocation is abolished if it is forced to interact with a nearby lysine or arginine. These results suggest key roles of the glutamic acid and the histidine copper ligand in the mechanism of proton translocation.

Photodissociation of fully reduced, CO-saturated cytochrome bo₃ causes ultrafast transfer of CO from heme iron to Cu_B in the binuclear heme iron-copper site. At low temperatures the CO remains bound to Cu_B for extended times. Here we show that the binding of CO to Cu_B perturbs the IR stretch of a carboxylic acid residue, which is identified as E286 by mutation to D or to C (FTIR data in Figure 2). The stretching frequencies of 1730 cm⁻¹ for E286 and 1760 cm⁻¹ for E286D suggest that both carboxylic acids are hydrogen-bonded, though more extensively in E286. In E286C this IR feature is lost altogether. We ascribe the frequency shifts in the carboxylic IR absorptions to the strengthened Lewis acid character of Cu_B upon binding of CO. Consequently, a histidine ligand is more strongly bound to Cu_B, and this effect is relayed to the 286 locus. Conversely, the 2065 cm⁻¹ CO stretch of Cu_B-CO is markedly affected by both mutations. CO binding to Cu_B also induces a downshift of an IR band attributable to a C-H stretch of histidine at about 3140 cm⁻¹. The results suggest that there is an easily polarizable, through-bond connectivity between one of the histidine copper ligands and the carboxylic group of E286. 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.

If a hydrogen-bonded water pathway were the conductive element between the histidine ligand of Cu_B and the carboxylic acid, in *both* the E286 and E286D cases, the following is a possible explanation for the inversion of the absorption shift in E286D. In such a scenario an upshift in frequency (as for E286) requires the carboxylic acid *-OH* group to be linked to the Cu_B ligand via hydrogen-bonding to the conduction path. In contrast, if it is the *C=O* group of the carboxylic acid that provides this link, then the carboxyl frequency is expected to be down-shifted (as found for E286D). The side chain configurations of the glutamic and aspartic acids in the 286 locus may thus differ by a 180° rotation around the terminal C-C bond. But if the side chain orientations of E286 and D286 are different, as suggested in the first scenario above, the inversion of the shift in E286D may simply be because the intrinsic sign of the through-covalent-bond effect is opposite to that of the through-hydrogen-bond effect, as well as weaker.

In either case, a hydrogen-bonded connectivity between E286 and a histidine copper ligand is inferred here. This is of obvious interest in view of the "histidine cycle" model of proton translocation by the heme-copper oxidases [7]. It may provide a key protonic connection, also proposed by [8], of this mechanism, which is "missing" from the

2.8 Å crystal structure. If so, it also implicates H334 in this mechanism, rather than H333 [8] or H284 [9] suggested previously.

Publications

1. S. Williams, T. P. Causgrove, R. Gilmanishin, K. S. Fang, R. H. Callender, W. H. Woodruff and R. B. Dyer, "Fast Events in Protein Folding: Helix Melting and Formation in a Small Peptide", *Biochemistry*, **35**, 691-7 (1996).
2. T. P. Causgrove and R. B. Dyer, "Picosecond Structural Dynamics of Myoglobin Following Photolysis of Carbon Monoxide", *J. Phys. Chem.*, **100**, 3273-7 (1996).
3. A. Puustinen, J. A. Bailey, R. B. Dyer, S. L. Mecklenburg, M. Wikstrom, W. H. Woodruff, "Fourier Transform Infrared Evidence for Connectivity Between Cu_β and Glutamic Acid 286 in Cytochrome *bo*₃ from E. Coli," *Biochemistry*, in press, (1997).
4. Y. Kim, G. T. Babcock, K.K. Surerus, J. A. Fee, R. B. Dyer, W. H. Woodruff, W. A. Oertling, "Cyanide Binding and Active Site Structure in Heme-Copper Oxidases: Normal Coordinate Analysis of Iron-Cyanide Vibrations of a₃²⁺CN- Complexes of Cytochromes *ba*₃ and *aa*₃." *Biospectroscopy*, in press, (1997).
5. A.P. Shreve, A. Parikh, A. Eberhardt, B.I. Swanson and R.B. Dyer, "Sum-Frequency Generation Studies of Self-Assembled Systems Using Multi-Channel Detection," Abs. Pap. Amer. Chem. Soc. 214 (pt. 2) (1997) 298-PHYS.
6. A.P. Shreve and R.B. Dyer, "A Versatile Ultrafast Laser System for Time-Resolved Infrared and Sum-Frequency Generation Spectroscopies," Rev. Sci. Instrum., in preparation.
7. S. Riistama, G. Hummer, A. Puustinen, R. B. Dyer, W. H. Woodruff, M. Wikstrom, "Bound Water in the Proton Translocation Mechanism of the Haem-Copper Oxidases," *FEBS Lett.*, **414**, 275-280 (1997).

References

- [1] X.D. Zhu, H. Suht and Y.R. Shen, *Phys. Rev. B* **35** (1987) 3047; A.L. Harris, C.E.D. Chidsey, N.J. Levinos and D.N. Loiacono, *Chem. Phys. Lett.* **133** (1987) 189; P. Guyot-Sionnest, J.H. Hunt and Y.R. Shen, *Phys. Rev. Lett.* **59** (1988) 1597; R. Superfine, J.H. Huang and Y.R. Shen, *Chem. Phys. Lett.* **144** (1991) 1.
- [2] Q. Du, R. Superfine, E. Freysz and Y.R. Shen, *Phys. Rev. Lett.* **70** (1993) 2313; Q. Du, E. Freysz and Y.R. Shen, *Phys. Rev. Lett.* **72** (1994) 238.
- [3] A.L. Harris and N.J. Levinos, *J. Chem. Phys.* **90** (1989) 3878; A.L. Harris and L. Rothberg, *J. Chem. Phys.* **94** (1991) 2449.
- [4] For examples, see T.P. Causgrove and R.B. Dyer, *Biochemistry* **32** (1993) 11985; M. Lim, T.A. Jackson and P.A. Anfinrud, *Proc. Natl. Acad. Sci. USA* **90** (1993) 5801, 8302; R. Diller, M. Iannone, R. Bolgomoni and R.M. Hochstrasser, *Biophys. J.* **60** (1991) 286.
- [5] For reviews see, Y.R. Shen, *Annu. Rev. Phys. Chem.* **40** (1989) 327; K.B. Eisenthal, *Annu. Rev. Phys. Chem.* **43** (1992) 627.
- [6] J.Y. Huang and A. Lewis, *Biophys. J.* **55** (1989) 835; J.Y. Huang, Z. Chen and A. Lewis, *J. Phys. Chem.* **93** (1989) 3314; Th. Rasing, J. Huang, A. Lewis, T. Stehlin and Y.R. Shen, *Phys. Rev. A* **40** (1989) 1684; Q. Song, C. Wan and C.K. Johnson, *J. Phys. Chem.* **98** (1994) 1999.
- [7] Morgan, J.E., Verkhovsky, M.I., Puustinen, A. & Wikström, M. (1995) *Biochemistry* **34**, 15633-15637.
- [8] Iwata, S., Ostermeier, C., Ludwig, B. & Michel, H. (1995) *Nature* **376**, 660-669.
- [9] Morgan, J.E., Verkhovsky, M.I. & Wikström, M. (1994) *J. Bioenerg. Biomembr.* **26**, 599-608.

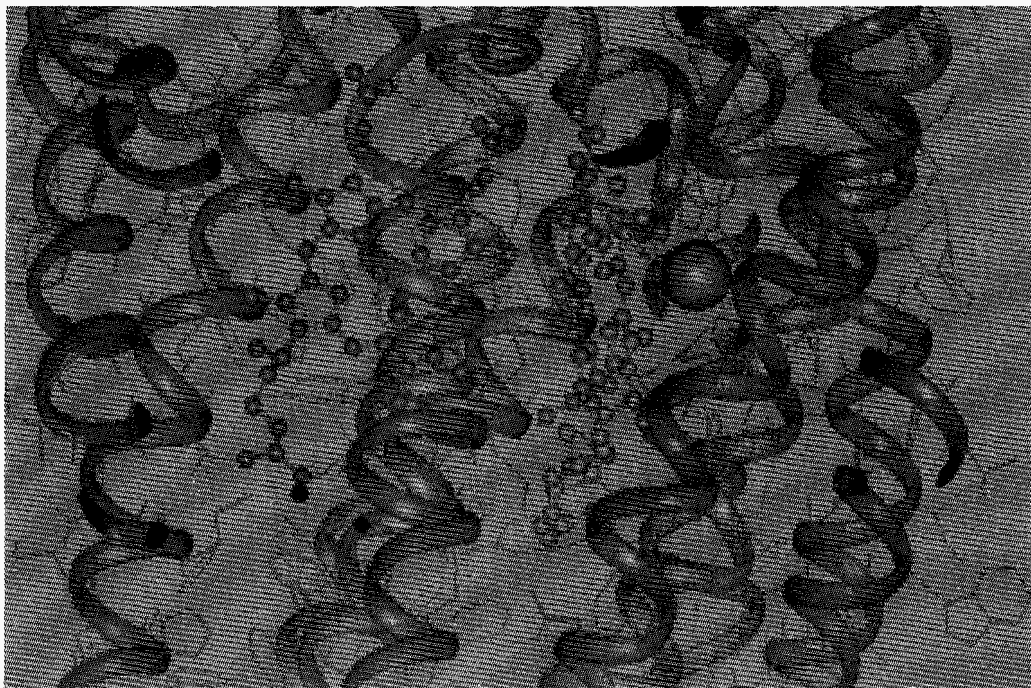


Figure 1. The crystal structure of CcO from bovine heart (Tsukihara et al., 1996). The view is a cut through the protein showing the two hemes and the Cu_β center. The putative proton pumping channel is in the bottom center of the structure, including the glutamic acid 286.

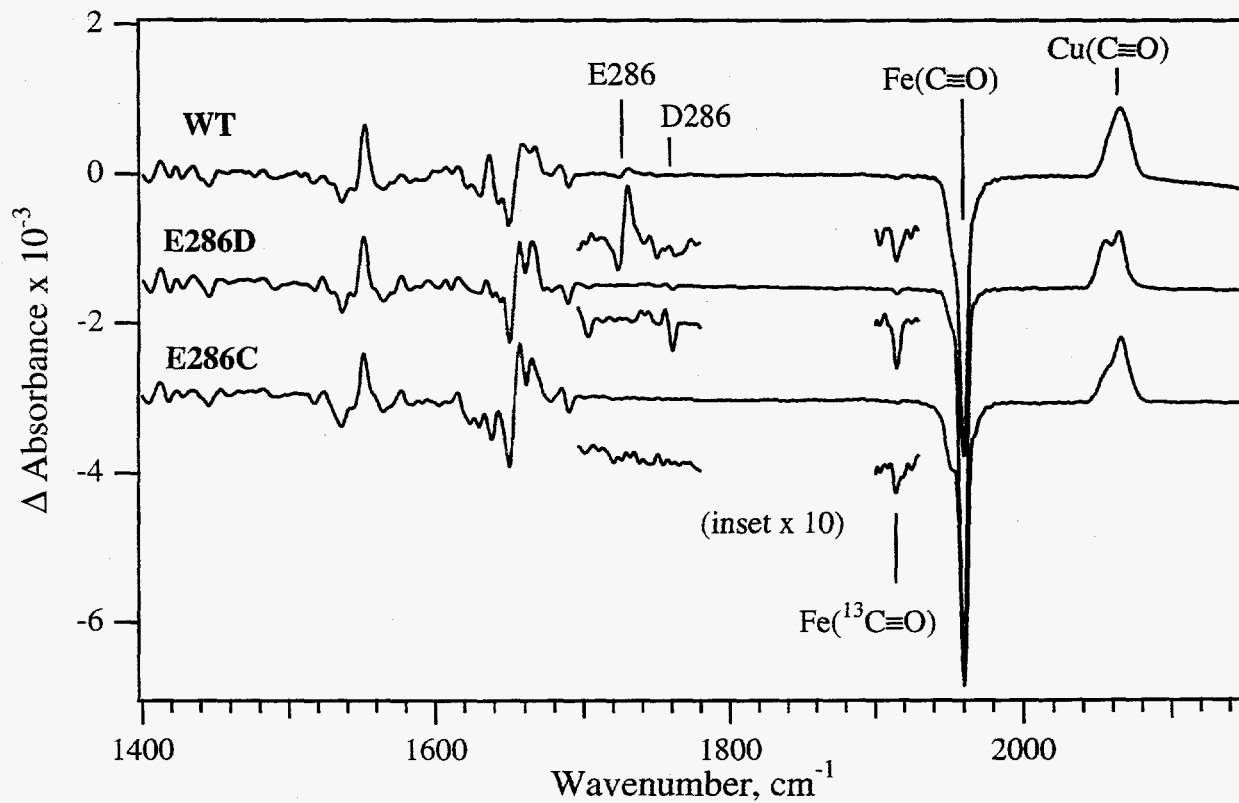


Figure 2. FTIR difference spectra (light minus dark) of photodissociated CcO-CO for wild-type and two mutants of glutamic acid 286. The change of ligation state of the metal centers (Cu-CO versus Fe-CO) is accompanied by changes in the protein, including the carboxylic acid vibrations of the glutamic acid side chain E286.