Journal of Bioenergetics and Biomembranes, Vol. 25, No. 2, 1993

Probing Heart Cytochrome *c* Oxidase Structure and Function by Infrared Spectroscopy

W. S. Caughey,[1] A. Dong,[1] V. Sampath,[1] S. Yoshikawa,[1,2] and X.-J. Zhao[1]

Received November 12, 1992; accepted November 19, 1992

IR spectra directly probe specific vibrators in bovine heart cytochrome *c* oxidase, yielding quantitative as well as qualitative information on structures and reactions at these vibrators. C—O IR spectra reveal that CO binds to $Fe^{2+}_{a_3}$ as two conformers each in isolated immobile environments sensitive to Fe_a and/or Cu_A oxidation state but remarkably insensitive to pH, medium, anesthetics, and other factors that affect activity. C—N IR spectra reveal that the one CN^- that binds to fully and partially oxidized enzyme can be in three different structures. These structures vary in relative amounts with redox level, thereby reflecting dynamic electron exchange among Fe_a, Cu_A, and Cu_B with associated changes in protein conformation of likely significance in O_2 reduction and H^+-pumping. Azide IR spectra also reflect redox-dependent long-range effects. The amide I IR bands, due to C—O vibrators of peptide linkages and composed of multiple bands derived from different secondary structures, reveal high levels of α-helix (~60%) and subtle changes with redox level and exposure to anesthetics. N_2O IR spectra reveal that these anesthetic molecules at clinically relevant levels occupy three sites of different polarity within the enzyme as the enzyme is reversibly, but only partially, inhibited.

KEY WORDS: Cytochrome *c* oxidase; infrared; carbon monoxide; cyanide; azide; secondary structure; anesthetics; nitrous oxide.

INTRODUCTION

This review is a response to the editor's request for an overview of those insights into the structure, properties, and functions of bovine heart cytochrome *c* oxidase (CcO)[3] that have resulted from use of infrared spectroscopy. Space limitations restrict coverage to work done mainly in our laboratory and to a representative, by no means comprehensive, list of references. A particularly important omission is the area of time-resolved and ultrafast IR studies of Woodruff and colleagues (Stoutland *et al.*, 1992). Such a review is timely in that infrared studies have by now provided much new information that is often only accessible by infrared methods. IR spectra of several types have proven useful. Spectra of metal-bound ligands (CO, CN^-, N_3^-) provide direct evidence of (1) which metal as well as the oxidation state of the metal that binds the ligand, (2) the effects of overall redox level and long-range intramolecular interactions on the ligand site, (3) the structure and dynamics of the O_2 reduction site environment, and (4) the effects of temperature, pH, medium, and other

[1]Department of Biochemistry, Colorado State University, Fort Collins, Colorado 80523.

[2]Permanent address: Department of Life Sciences, Himeji Institute of Technology, 1479 Kamigohri, Akoh, Hyogo, 678-12, Japan.

[3]The abbreviations and trivial names used are: CcO, cytochrome *c* oxidase; CcO(IV), CcO(III), CcO(II), CcO(I), and CcO(0) represent cytochrome *c* oxidase at different overall redox levels, the fully oxidized, one-, two-, three-, and four-electron reduced species, respectively; Fe_{a3}, the iron that most readily binds external ligands; Cu_B, the copper most closely associated with Fe_{a3}; Fe_a and Cu_A, the iron and copper considered to be involved in the transfer of electrons from cytochrome *c* to the O_2 reduction site; C—O IR bands and C—N IR bands, infrared bands due to the stretching vibrations of carbon monoxide and cyanide, respectively.

0145-479X/93/0400-0081$07.00/0

perturbations on ligand binding. New methods of measurement and analysis of amide I spectra have made possible the determination of the secondary structures present in CcO and detection of subtle changes in secondary structure that may, or may not, result from changes in such factors as redox level, ligand binding, medium, and exposure to anesthetics. Nitrous oxide, an anesthetic that inhibits CcO partially and reversibly, has been shown, in terms of its IR spectra, to occupy sites within the protein. The azide anion has also been shown to occupy nonmetal sites within CcO protein. The nitrous oxide IR spectrum is also useful as a means of determining the product formed when CcO acts as a nitric oxide reductase (X.-J. Zhao, V. Sampath, and W. S. Caughey, unpublished data). Similarly the CO_2 IR spectrum has been used in studies of the carbon monoxide dioxygenase activity of CcO (Young and Caughey, 1986).

INFRARED STUDIES OF LIGANDED CARBON MONOXIDE

Carbon monoxide generally, but not always, binds to the same sites in metalloproteins that bind O_2. As a result, CO enjoys a rich literature of comparative biochemistry with O_2. Furthermore, CO represents a very effective infrared active probe of O_2-binding sites because the frequencies and bandwidths of C—O stretch bands are highly sensitive to the bonding and environment of the CO ligand. Therefore, these band parameters can give valuable information on structures at the sites of CO and O_2 binding (Caughey, 1980).

Nature of the C—O IR Bands

The first IR spectra of CcO to be measured (Caughey *et al.*, 1970) and now the most extensively studied CcO infrared spectra are those of metal-bound CO (Einarsdottir *et al.*, 1988). The absorption due to the C—O stretching vibrations of CcO(0)CO, the carbonyl of fully reduced enzyme, is shown in Fig. 1. Here the spectrum was obtained with an FTIR spectrometer and a solution of crystalline bovine heart enzyme with Brij 35 as detergent (Yoshikawa *et al.*, 1991) in phosphate buffer pH 7.4 at 20°C. The high signal-to-noise ratio (S/N) permits accurate determination of the band parameters: the frequency (ν_{CO}), the half band width ($\Delta\nu_{1/2}$), and the intensity. Achieving a high S/N is facilitated by the location of the CO IR band within a region of the spectrum where the

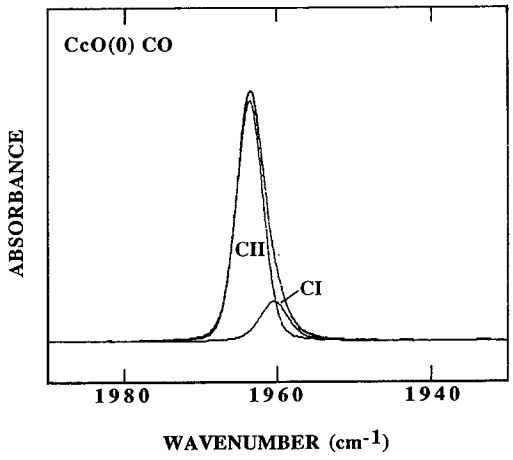

Fig. 1. The CO IR spectrum of fully reduced bovine heart cytochrome *c* oxidase carbonyl and curve-fitted deconvolution. Spectrum was obtained with a solution of crystalline enzyme at a heme concentration of 1 mM in 10 mM sodium phosphate buffer pH 7.4, at 20°C. Curve-fitting deconvolution was performed on a 386-based personal computer using Spectra Calc software (Galactic Industries Corp.) with 80% Gaussian and 20% Lorentzian profiles. CI: ν_{CO} 1960 cm^{-1}, $\Delta\nu_{1/2}$ 4.5 cm^{-1}, and 17% of total band area; CII: ν_{CO} 1963.6 cm^{-1}, $\Delta\nu_{1/2}$ 4.1 cm^{-1}, and 83% of total band area.

water spectrum has a "window," i.e., a region of relatively low absorbance (Fig. 2). The observed absorbance of Fig. 1 can be accurately deconvoluted into two bands, CI and CII, with the band parameters 1960 and 1963 cm^{-1}, respectively. Each band is extraordinarily narrow ($\Delta\nu_{1/2} \sim 4\,cm^{-1}$ compared to the

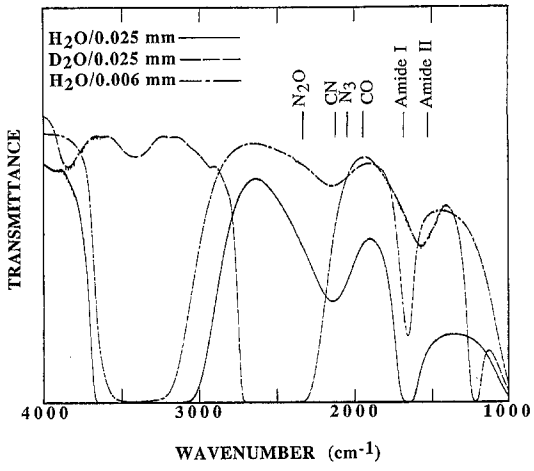

Fig. 2. Infrared spectra of H_2O vs. air and D_2O vs. air recorded in transmission mode. Approximate wavenumber locations of absorption bands due to some ligands bound to heme iron, anesthetic N_2O bound to protein, and amide I and II groups are indicated.

8–20 cm^{-1} found in other hemeprotein carbonyls) (Einarsdottir *et al.*, 1988; Potter *et al.*, 1990).

Nature of the $CO(O_2)$ Binding Site in Fully Reduced CcO

The discovery of ν_{CO} at 1963 cm^{-1} gave the first direct evidence that CO binds stably to Fe^{2+}, and not to Cu^{+}, within an environment well isolated from external medium (Caughey *et al.*, 1970). If CO was bound to Cu^{+}, a ν_{CO} about 100 cm^{-1} greater was expected; later such a band was found for the transient Cu^{+}CO complex formed following photodissociation of CO from ferrous heme a_3 (Fiamingo *et al.*, 1982). A ν_{CO} of 1963 cm^{-1} was recognized as fully consistent with CO binding to a reduced heme A trans to a histidine in view of the ν_{CO} values found for model carbonyl complexes of hemes A and B as well as for myoglobin and hemoglobin carbonyls (Alben and Caughey, 1968; Potter *et al.*, 1990). In another early IR study the quantitative transfer of CO from CcO(0)CO to human hemoglobin A provided the first direct evidence that CO binds stably to only one metal, Fe_{a3}, and not to any other metal center, a finding long anticipated but not then proven (Yoshikawa *et al.*, 1977). The similarities of ν_{CO} values for CO bound to CcO(0), hemoglobins, and myoglobins also gave strong support for O_2 binding to Fe^{2+} in CcO in a bent-end-on stereochemistry, a stereochemistry first identified in O_2 infrared spectra of oxyhemoglobins and oxymyoglobins (Caughey *et al.*, 1975; Potter *et al.*, 1987). Similar O_2 to Fe^{2+} bonding in CcO and the globins is now strongly supported in recent resonance Raman studies (Ogura *et al.*, 1990; Varotsis *et al.*, 1990; Han *et al.*, 1990).

The much narrower CO band of CcO(0)CO compared with hemoglobin A carbonyl (and all other hemeprotein carbonyls) demonstrated a distinct difference between the CO (and O_2) binding sites in the two proteins (Einarsdottir *et al.*, 1988). Also carbonyl complexes of isolated heme A and other hemes in solution exhibit much wider CO bands; both ν_{CO} and $\Delta\nu_{1/2}$ values vary widely among different solvents. A $\Delta\nu_{1/2}$ value as small as 4 cm^{-1} is clearly inconsistent with exposure of the bound CO to external aqueous medium. Such a narrow band can only result if, in the population of C–O vibrators, the vibrators experience nearly identical immediate environments. For this to occur, the environment adjacent to the CO ligand must be unusually immobile, much less mobile than is the case for all other hemeprotein sites for which CO infrared spectra are known and for heme A carbonyls in solution.

Another characteristic of the ligand binding site is revealed by the presence of two CO stretch bands. Two bands (CI and CII) indicate there are two populations of CO vibrators. Both populations exhibit $\Delta\nu_{1/2}$ values of about 4 cm^{-1} and, therefore, within a given population all the CO ligands experience similar environments. The cause of the small (3 cm^{-1}) difference in ν_{CO} between the CI and CII bands undoubtedly results from two discrete protein conformers in which the CO environment and/or Fe–C–O bonding are different. Multiple CO IR bands at a single hemeprotein ligand site are commonly encountered. These phenomena have been most extensively studied in hemoglobin and myoglobin carbonyls where four bands are usually found with each band representing a discrete protein conformer (Caughey *et al.*, 1981; Potter *et al.*, 1990). Furthermore, the four globin conformers interconvert at a rate greater than the NMR time scale but less than the infrared time scale. Thus, the CI and CII bands of CcO(0)CO are expected to represent discrete protein conformers that interconvert rapidly. The approximately fivefold greater intensity of band CII than band CI indicates CII is the more stable conformer under conditions such as those of Fig. 1.

Redox Level Effects on CO Bonding

The overall redox level of the enzyme alters the ν_{CO} of bound CO. Exposure of an anaerobic solution of fully oxidized CcO, i.e., CcO(IV), to CO promotes slow reduction of the enzyme with formation of a C–O IR band at 1965.5 cm^{-1}. Upon further reduction either chemically (e.g., with NADH and catalytic amounts of PMS; Yoshikawa and Caughey, 1982) or electrochemically (E. O. Dodson, X.-J. Zhao, C. M. Elliott, and W. S. Caughey, unpublished data) ν_{CO} remains near 1965.5 cm^{-1} until about two reducing equivalents have been added. Further reduction causes a shift in ν_{CO} to $\sim$ 1963.5 cm^{-1}. The two CI and CII bands are present at both high and low states of reduction with the difference in ν_{CO} as well as the band widths remaining the same. CO serves as a ligand only to reduced Fe_{a3}. However, the oxidation states of the other redox active metals, Fe_a, Cu_A, and Cu_B, can obviously influence CO bonding to $Fe^{2+}_{a_3}$, but only to the extent of a 2 cm^{-1} shift in ν_{CO} with no effect on $\Delta\nu_{1/2}$ or on the relative amounts of CI and CII. This long-range effect of other center(s) on the O_2 reaction site

appears relevant to CcO function and is of particular interest concerning possible structural mechanisms of respiratory control and proton pumping.

The shift in ν_{CO} from 1965.5 to 1963.5 cm^{-1} without an accompanying shift in $\Delta\nu_{1/2}$ that occurs during the addition of electrons to the enzyme is most simply explained, based on evidence from infrared studies with hemeprotein and model heme carbonyls, as the result of alterations in strength of bonding between the proximal histidine and Fe_{a3} while keeping the distal area constant. This explanation would require that the environment about the CO ligand remains the same in both 1965.5 and 1963.5 cm^{-1} structures, including keeping Cu_B as well as Fe_{a3} reduced, whenever CO is bound. Keeping the distal area constant is consistent with unchanged $\Delta\nu_{1/2}$ and CII/CI values. Model CO IR studies indicate it is unlikely for such a small shift in ν_{CO} and no shift in $\Delta\nu_{1/2}$ to occur when there is a change in the oxidation state of the Cu_B adjacent to bound CO (Einarsdottir *et al.*, 1988). However, it is possible that any differences in the interactions of CO with its environment in Cu^+ versus Cu^{2+} oxidation states are reduced by maintenance of an essentially constant effective charge at Cu_B by loss of a proton from a ligand (e.g., histidine imidazole) upon oxidation of Cu^+ to Cu^{2+} and by structures at Cu_B that minimize the extent of change in ligand stereochemistry (Yoshikawa and Caughey, 1990). Model studies show the 2 cm^{-1} difference in ν_{CO} can be readily achieved if a change in the oxidation state of Fe_a caused a relatively modest conformational change in the protein (e.g., across helix X; Hosler *et al.*, 1993) that altered the bonding of a putative histidine to Fe_{a3}. Such a long-range effect of Fe_a oxidation state on CO bonding to Fe_{a3} is an especially attractive explanation of the shift in ν_{CO} if the reduction of CcO(IV) by two electron equivalents in order to permit *both* Fe_{a3} and Cu_B to become reduced is required before CO (and presumably O_2) can bind to reduced Fe_{a3}. Earlier infrared evidence suggested that only one electron need enter CcO(IV) for the 1965.5 cm^{-1} band to be observed (Yoshikawa and Caughey, 1982). Unfortunately the experimental problems associated with accurately monitoring the extent of autoreduction induced by CO (Young and Caughey, 1987) render somewhat equivocal the degree of reduction needed to permit CO binding. However, it is unequivocal that once CO is bound to partially reduced CcO the only significant change in the C–O IR band upon further reduction is a 2 cm^{-1} shift in ν_{CO}. Clarification of the structural basis for this 2 cm^{-1} shift in ν_{CO} can be expected to provide further insight into the control of enzyme function as redox level changes occur.

Temperature-Induced Denaturation

The C–O IR bands provide a sensitive means for detection of changes in structure, including denaturation, at the O_2 reduction site. Instability in these bands with only modest elevations in temperature has been observed in solutions of CcO(0)CO in sodium phosphate buffers at pH 7.4 (Einarsdottir *et al.*, 1988). At temperatures below 24°C the spectral changes with temperature were small and fully reversible. For example, a temperature difference spectrum (4° minus 24°C) contained a positive band at 1964 cm^{-1} (CII) and a negative band at 1960 cm^{-1} (CI). Thus, as expected for a mixture of two protein conformers in equilibrium, the less stable conformer CI is in relatively greater amount at the higher temperature. Upon increasing the temperature to 37°C, two minor bands at 1969 and 1955 cm^{-1} intensified, the 1963 cm^{-1} band weakened, and a new broad band developed at 1973 cm^{-1}. After these spectral changes had occurred, bringing the solution back from 37 to 24°C did not fully restore the spectrum originally observed at 24°C. Longer standing at 37°C or raising the temperature (e.g., to 52°C) resulted in a broad absorbance ($\Delta\nu_{1/2} \sim 25\,cm^{-1}$) centered at 1970–1975 cm^{-1} which remained unchanged when the temperture was reduced to 24°C. The formation of a very broad CO band represents a gross disorganization of the structure at the O_2 reaction site. The CO remains bound to heme Fe^{2+}, but the immediate environment of the CO ligand has gone (irreversibly) from one that is unusually immobile to a very mobile environment wherein the population of C–O vibrators experience as wide a range of different environments as would occur upon at least partial exposure of the heme-bound CO to the external aqueous medium. However, at 37°C the changes in visible/Soret as well as C–O IR spectra occur slowly (over 2–3 days). Thus, under these conditions, the loss of native ligand site structure is gradual rather than abrupt.

pH Effects on the CO Binding Site

Since the oxidase activity is highly sensitive to changes in pH, any effects of pH on the structure of the O_2 reduction site could help explain the changes in activity. Therefore the CO infrared bands of CcO(0)CO over the pH range from 4.4 to 11.4 were measured (Fig. 3) (Einarsdottir *et al.*, 1988). Between

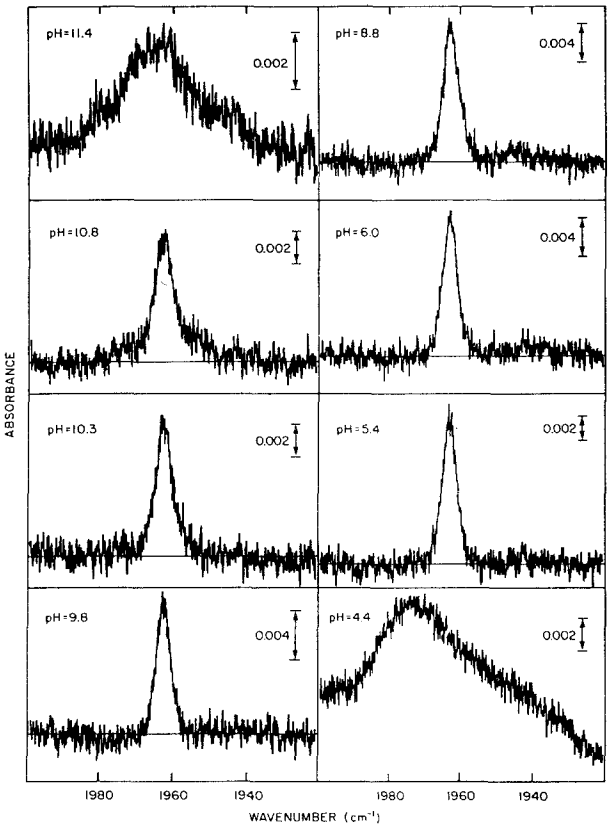

Fig. 3. Effects of pH on C–O infrared spectra of fully reduced bovine heart cytochrome *c* oxidase in 10 mM sodium phosphate buffer at 24°C. From Einarsdottir *et al.* (1988).

pH 5.4 and 10.8 the infrared absorbance can be deconvoluted into two bands CI ($\sim 1959\,cm^{-1}$) and CII ($\sim 1963\,cm^{-1}$) with no significant changes with pH for either ν_{CO} or $\Delta\nu_{1/2}$ of these bands. However, it was noted that CI intensified slightly relative to CII as the pH was raised from 5.4 to 9.8. Intensification of CI became somewhat more pronounced from pH 10 to 11. Below pH 5 and above pH 11 the CO spectra contained broad bands ($\Delta\nu_{1/2} \sim 20\,cm^{-1}$) centered at 1973 and $1965\,cm^{-1}$ at pH 4.4 and 11.4, respectively. Returning the solution to neutrality from either pH extreme did not alter the CO infrared spectrum. Therefore, an irreversible change in CO binding site structure occurred at both pH extremes. The lack of shifts in either ν_{CO} or $\Delta\nu_{1/2}$ for CI and CII over the pH range 4.5–10.8, a range over which large changes in oxidase activity occur, indicates that little pH-induced stereochemical changes are occurring at the ligand site. However, it may be relevant that the CII/CI intensity ratios were greatest at pH 5.4 and 6.0 and the plots of pH versus oxidase activity had two sharp peaks of maximum activity centered at pH 5.6–5.7 and 6.0 (Einarsdottir *et al.*, 1988). The structural basis of the effects of pH on the CII/CI ratio remains unclear. However, ionizable groups close to bound $CO(O_2)$ appear either absent or totally isolated from the external medium.

Medium Effects on the CO Ligand Site

The high sensitivity of the C–O IR band parameters to changes in bonding and environment permits the monitoring of any potential perturbation at the $CO(O_2)$ reaction site. In addition to the studies of temperature and pH effects discussed above, several other factors that affect oxidase activity have been examined to determine if effects on the O_2 reaction site structure can provide an explanation for the effects on activity. Changes in medium by exchange of D_2O for H_2O or by addition of cosolvents such as dimethyl sulfoxide, glycerol, and ethylene glycol can markedly reduce oxidase activity without apparent alterations in C–O IR bands (Einarsdottir *et al.*, 1988; X.-J. Zhao, and W. S. Caughey, unpublished data). Thus, based on IR evidence, these medium effects on activity appear due to factors other than perturbations of the ligand site structure.

INFRARED STUDIES OF LIGANDED CYANIDE

Cyanide, as well as CO, is an infrared active ligand that binds to CcO and is a potent inhibitor of respiration. C–N IR spectra have proven useful as a probe of ligand sites in iron porphyrins, CcO, and other hemeproteins (Yoshikawa *et al.*, 1985; Yoshikawa and Caughey, 1990). CN^- is less selective in binding to metal ions than is CO. CN^- may bind to many different metals and to different oxidation states of a given metal. However, the accurate determination of C–N IR spectra is in several respects more difficult than with C–O IR spectra. Whereas unliganded CO in solution is not observed in IR spectra, both HCN and unliganded CN^- anion are seen as bands at 2093 and $2079\,cm^{-1}$, respectively, within regions where metal-bound CN^- bands also appear (Yoshikawa *et al.*, 1985). Replacement of H_2O with D_2O reduces the HCN problem since ν_{CN} for DCN is at $1887\,cm^{-1}$ but introduces the problem of high variable absorbance by D_2O in the C–N IR region of the spectrum (Fig. 2). Furthermore, contaminant metal ions can also give cyanide IR bands which can be helpful for

their detection but can also be a nuisance. Nevertheless, very insightful CN^- spectra have been obtained for CcO in D_2O media.

The first C–N IR study for CcO carried out with a dispersive infrared spectrometer considered CN^- binding to CcO at five redox levels from IV to 0. Redox dependent metal C–N IR bands were detected and tentative assignments given without benefit of knowing the number of CN^- that could bind at one time (Yoshikawa and Caughey, 1990). Recently this area was reinvestigated with the benefit of the increased sensitivity of an FTIR instrument and a crystalline enzyme preparation that permitted more accurate measurements of C–N IR bands as both the CN^- concentration and the redox levels from IV to I were varied (S. Yoshikawa, X.-J. Zhao, and W. S. Caughey, unpublished data). Over the IV to I overall redox level range three C–N stretch bands were found: A $\sim$ 2151 cm^{-1}, B $\sim$ 2131 cm^{-1}, and C $\sim$ 2090 cm^{-1}. The relative intensities of the A, B, and C bands were highly dependent on redox level but were, at any given redox level, independent of CN^- concentration. Only A was present at level IV, A, B, and C were found at levels III and II, and only B and C at level I. Thus, the intensities of B and C increased and A weakened with progressive reduction of the enzyme. The high affinity of fully and partially oxidized levels of the enzyme for CN^- permitted demonstration that, at a given redox level, while increasing the CN^- concentration from 0.3 to more than equal the heme a_3 concentration, the relative intensities of bands A, B, and C remained constant and the bands attained maximum intensities when the CN^- concentration became equivalent to the Fe_{a3} concentration. Thus, only one CN^- can and needs to bind to give rise to the three different structures represented by A, B, and C with substantially different ν_{CN} values. Furthermore, these structures can be sufficiently similar in energy to exist simultaneously with the relative amounts dependent upon the overall redox level but independent of the extent of CN^- binding.

These findings raise the intriguing question: What is the nature of the structural differences between A, B, and C that result in three widely different ν_{CN} values? Based on reference to model compounds and the assumption (now known to be invalid) that two CN^- ligands can bind at the same time, band assignments of $Cu^{2+}CN$ at 2151 cm^{-1}, $Fe^{3+}CN$ at 2131 cm^{-1}, $Cu^{+}CN$ at 2093 cm^{-1}, and $Fe^{2+}CN$ at 2058 cm^{-1} were made (Yoshikawa and Caughey, 1990). The first three assignments indeed may possibly still approximate the bonding in A, B, and C, respectively. However, consideration of all the evidence now available provides strong support for $Fe^{3+}CN$ bonding in all three A, B, and C structures. Soret spectra support a low-spin $Fe^{3+}_{a_3}$ in A, B, and C, a finding consistent with, if not compelling, for $Fe^{3+}_{a_3}CN$ in each structure. The high resistance of CcO(I)CN to reduction to CcO(0)CN also suggests CN^- binding to $Fe^{3+}_{a_3}$. Furthermore, the unusually high immobility and stability of the environment about Fe_{a3}-bound CO that is detected in C–O IR spectra may make the assumption of a single similar site for the CN^- ligand in each A, B, and C site appear more reasonable.

The assumption of CN^- binding to $Fe^{3+}_{a_3}$ in A, B, and C still raises the question: How can such wide variations in ν_{CN} occur? Evidence from model studies indicate that the observed ν_{CN} differences are much too large to result from a trans effect, i.e., from changes in bonding to $Fe^{3+}_{a_3}$ trans to the bound CN^- (Yoshikawa *et al.*, 1985). However, there is ample evidence from other model studies that variations in ν_{CN} as large as those observed can result from variations in the distal environment adjacent to the N of $Fe^{3+}CN$ (Fernandez Bertran *et al.*, 1990a,b). Thus, the changes in ν_{CN} could reasonably be the sole result of the CN^- ligand in A, where ν_{CN} is 2151 cm^{-1}, experiencing a more positive distal environment than the CN^- of B with ν_{CN} of 2131 cm^{-1} and the CN^- of C with ν_{CN} at 2090 cm^{-1}, an even less positive (more negative) distal environment than in B. An obvious factor influencing distal polarity is the oxidation state of Cu_B; Cu_B must be oxidized in level IV and reduced in level I, representing a change in polarity consistent with the changes in ν_{CN} from A to C. Another characteristic of the CN^- ligand is the high avidity of the terminal N for accepting a proton in H-bonding (Yoshikawa *et al.*, 1985). The bent-end-on O_2 ligand also likes to participate in H-bonding, whereas the CO ligand does not. The enzyme must provide hydrogens adjacent to bound O_2 to enable O–H bonds to form during O_2 reduction (Einarsdottir *et al.*, 1988). Space restrictions make the imidazoles of putative Cu-bound histidines likely H-donors (Surerus *et al.*, 1992). These factors suggest that the following may illustrate the structural differences in A, B, and C:

A	$Fe^{3+}CN\cdots HImCu^{2+}$	ν_{CN} 2151 cm^{-1}
B	$Fe^{3+}CN\cdots HImCu^{+}$	ν_{CN} 2131 cm^{-1}
C	$Fe^{3+}CN\cdots {}^{-}ImCu^{+}$	ν_{CN} 2090 cm^{-1}

The lower ν_{CN} in B than A can result from stronger H-bonding in A than B. The ν_{CN} of 2131 cm^{-1} is

consistent with ν_{CN} found for hemeprotein cyanides where H-bonding to CN occurs (Yoshikawa *et al.*, 1985). The large decrease in ν_{CN} from B to C cannot be related to a change in the oxidation state of Cu_B since both B and C are present in redox level I where all the Cu_B must be reduced. However, such a decrease could result from deprotonation of the imidazole or possibly another protic ligand which could not only remove H-bonding between the Cu_B^+ bound ligand and CN^- but would introduce a negatively charged center near the CN^-. Model studies suggest that simple removal of H-bonding would not be sufficient to reduce ν_{CN} from 2131 to 2090 cm^{-1}, whereas introduction of a nearby negative charge could cause such a shift. Thus, B and C can represent two conformations of nearly equivalent energy where interconversion between the two conformations involve proton transfers to or from another group (X). Here X would accept a proton from B, allowing C to form, and XH would donate a proton to C to form B. One may speculate further than acceptance of a proton from B by X can promote loss of a proton elsewhere via a process related structurally to proton pumping. The likely participation of Cu_B in proton pumping has been discussed (Yoshikawa and Caughey, 1982, 1990, 1992; Babcock and Wikstrom, 1992). It should be noted that this mode of CN^- binding for the heart enzyme is distinctly different from the reported $Fe^{2+}_{a_3}$C-N$\cdots Cu^{2+}_B CN$ structure for the cytochrome ba_3 from *Thermus Thermophilus* that results upon treatment of the fully oxidized enzyme with a large excess of CN^- (Surerus *et al.*, 1992).

In summary, C–N IR spectra have revealed several new facets of CcO structure–property interrelationships. At fully and partially oxidized redox levels (i.e., IV $\leftrightarrow$ I) only one CN^- binds per Fe_{a3} with high affinity. In the fully reduced enzyme, which binds CN^- with lower affinity, the number of cyanides that bind is not yet established. The binding of one CN^- to partially oxidized states (III, II, and I) maintains Fe_{a3} in the ferric oxidation state and results in multiple C–N stretch bands, three bands for III and II, and two for I. Only one band appears in level IV. The three types of C–N IR bands represent three different structures at the CN^- binding site(s). The relative stabilities of the three structures, which can be quite similar, are dependent upon the overall redox level of the enzyme but not on CN^- concentration. Their structural differences must result from differences in the oxidation states of the Fe_a, Cu_A, and Cu_B redox centers and any associated changes in protein conformation. Such structural differences and redox-dependent effects are likely to be relevant to the O_2 reduction and proton pumping functions of the enzyme.

INFRARED STUDIES OF AZIDE BINDING

The azide anion is another infrared active ligand that inhibits CcO activity and respiration. Bands of N_3^- are found between 2000–2100 cm^{-1} within the water window (Fig. 2). In contrast to CO and CN^-, N_3^- binds only to oxidized Fe or Cu ions. The one IR study of CcO N_3^- complexes was limited by the sensitivity of a dispersive spectrometer and the lack of information on the stoichiometry of N_3^- binding (Yoshikawa and Caughey, 1992). Nevertheless, many new observations were made, raising several interesting questions concerning the structural implications of N_3^- IR spectra. The effects on IR spectra of the introduction of ^{15}N into N_3^- gave evidence that azide can occupy nonmetal ion sites within the protein as well as bind to a metal ion. Bands for metal-ion bound N_3^- were found at $\sim$2040 cm^{-1} at redox levels IV to I, at 2016 cm^{-1} at III to I, and at 2004 cm^{-1} only in III. Reasonable assignments based on the available information were Cu^{2+}_B at $\sim$2040 cm^{-1} and $Fe^{3+}_{a_3}$ at 2016 and 2004 cm^{-1}. The 2016 and 2004 cm^{-1} bands of $Fe^{3+}_{a_3}N_3$ were suggested to arise from long-range effects of the difference between $Fe^{2+}_a\cdots Cu^{2+}_A$ and $Fe^{3+}_a\cdots Cu^{+}_A$ assuming that two azides could bind simultaneously to $Fe^{3+}_{a_3}$ and Cu^{2+}_B.

Recent observations make some of the original interpretations of these N_3^- IR bands open to question although they may still be correct. For example, the recent CN^- titration experiments discussed above indicate that only one CN^- can enter the Fe_{a3}–Cu_B site at a time. So, if only one CN^- can enter, is it reasonable to expect two azides to enter, especially when N_3^- is larger than CN^-? Additionally, detailed second-derivative analysis of the effects of excess N_3^- on the Soret spectrum of CcO(IV) indicates no perturbation of the heme a_3 spectrum by N_3^-, but a small shift in the heme *a* spectrum could be detected. Does the N_3^- bind to Cu^{2+}_B and thereby cause an effect on the Soret spectrum of heme *a* or does N_3^- bind directly to Fe^{3+}_a? In this regard, Li and Palmer have recently obtained EPR evidence that N_3^- at high concentrations can bind to oxidized heme *a* (W. Li and G. Palmer, personal communication). Therefore, should the 2040 cm^{-1} band be assigned to $Fe^{3+}_a CN$ and the 2016 and

2004 cm^{-1} bands to $Fe^{3+}_{a_3}$ CN? It appears obvious that azide binding to CcO merits further study.

PROTEIN SECONDARY STRUCTURE FROM AMIDE I SPECTRA

Determination of the secondary structures of protein in water solutions by infrared spectroscopy has become well established (Susi and Byler, 1986; Dong *et al.*, 1990, 1992a,b). Since IR is not subject to lipid-induced light scattering artifacts, as is the case with circular dichroism, it is receiving increased attention for studies of membrane protein structure (Mendelsohn and Mantsch, 1986). The most useful IR band for the direct measurement of secondary structure is a broad band found between 1700 and 1620 cm^{-1}. This band, the amide I band, arises almost exclusively from the C—O vibration of the peptide linkages of the protein chain (Miyazawa and Blout, 1961; Krimm and Bandekar, 1986). The overall amide I band contour is a result of the superposition of up to 14 discrete bands each of which is characteristic of a specific type of secondary structure (Dong *et al.*, 1990; A. Dong, P. Huang, B. Caughey, and W. S. Caughey, unpublished data). By use of second-derivative analysis the components ascribed to α-helix (band at 1656 ± 2 cm^{-1}), β-sheet (multiple bands between 1698–1689 cm^{-1} and 1642–1623 cm^{-1}), turn (multiple bands between 1688 and 1666 cm^{-1}), and random (band at 1648 ± 2 cm^{-1}) structures can be resolved. A recent IR study in our laboratory of 26 globular proteins with widely varying secondary structure compositions known from crystal structures gave strong support to the quantitative and qualitative validity of these assignments. The maximum absorbances of the amide I band of proteins with predominantly α-helix structure were found near 1656 ± 2 cm^{-1} and those with predominantly β-sheet structures between 1643 and 1631 cm^{-1} (A. Dong, P. Huang, B. Caughey, and W. S. Caughey, unpublished data).

Secondary Structure of CcO(0)CO

The primary spectra of solutions of crystalline bovine heart CcO as the fully reduced carbonyl exhibit a maximum amide I absorbance near 1657 cm^{-1}, thereby providing that the secondary structure is predominantly α-helix (Fig. 4, upper panel). The spectrum of Fig. 4 also shows a strong band at 1547 cm^{-1}, the amide II band, and a very weak band at 1964 cm^{-1} due to bound CO. A second-derivative analysis of the amide I band revealed components due to α-helix (1657 cm^{-1}), β-sheet (1696, 1640, and 1631 cm^{-1}), turn (1686, 1680, and 1675 cm^{-1}), and random structures (1652 cm^{-1}, detected by further curve-fitting deconvolution) (Fig. 4, lower panels). Quantitative analysis confirmed that α-helix represented more than 60% of the secondary structure, whereas β-sheet, turn, and random structures were in lesser amounts (Table I). Secondary structure compositions obtained from CD studies are also listed in Table I. The CD results

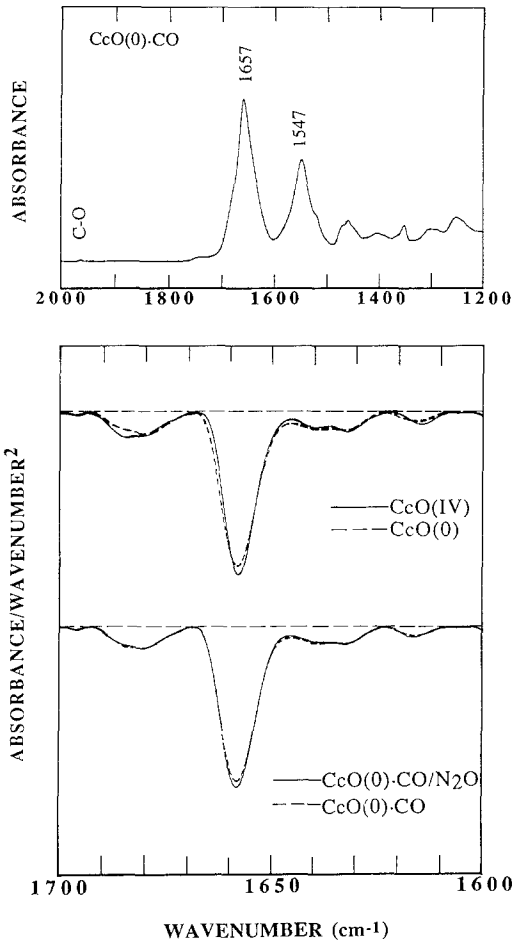

Fig. 4. Primary and second-derivative spectra of solutions of crystalline bovine heart cytochrome *c* oxidase in 10 mM sodium phosphate buffer, pH 7.4, at 20°C. (Top panel) Primary spectrum of fully reduced CcO carbonyl. The contributions of the buffer and water vapor have been subtracted from the primary spectrum. (Bottom panel) Comparisons of the second-derivative amide I bands of fully oxidized and fully reduced CcOs (upper spectra) and fully reduced CcO carbonyl with or without N_2O (lower spectra). The high spectral reproducibility of the second-derivative infrared method is shown by the superimposition of two independently measured spectra for each CcO form.

Table I. Secondary Structures of Bovine Heart Cytochrome *c* Oxidase in Water as Determined by Amide I Infrared Second-Derivative Analysis and by Circular Dichroism

Overall redox level	Secondary structure (%)				Method	Reference
	α-Helix	β-Sheet	Turn	Random		
CcO(IV)	61.0	13.0	15.4	10.6	IR[a]	This work
	39	–	–	–	CD	[b]
	36.8	17.7	13.5	30.0	CD	[c]
	63.0	0	23.0	14.0	CD	[d]
	44	13	12	31	CD	[e]
CcO(0)	63.2	14.2	13.4	9.2	IR[a]	This work
	44.0	–	–	–	CD	[b]

[a]Conditions as in Fig. 4.
[b]Myer (1971).
[c]Bazzi and Woody (1985).
[d]Park *et al.* (1992); estimated by the method of Chang *et al.* (1978).
[e]Park *et al.* (1992); estimated by the method of Perczel *et al.* (1989, 1991).

vary substantially. Only in one method of estimation did the α-helix content agree with the IR determination. The CD appears much less useful for the determination of secondary structures of membrane proteins than is the case with other proteins (Urry, 1972; Mendelsohn and Mantsch, 1986). The CD spectra of a membrane protein often exhibit some degree of distortion in shapes, intensities, and/or positions of CD bands due to optical artifacts of differential light scattering and differential absorption flattening (Urry and Long, 1980). Artifacts result in part from the n-π^* and π-π^* bands of the lipid ester moiety with similar bands in the peptide (10% tightly bound lipid will severely affect the accuracy of the protein conformation estimate by CD). Artifacts also result from an intensity decrease in the α-helix curve of a protein due to interactions between α-helices. For two helices separated by 7 to 10 Å the overall CD intensity of the α-helices could decrease by about 10%; with four interacting α-helices, more pronounced effects are expected (Manning, 1989). Since even crystals of bovine heart CcO contain substantial amounts of detergent (~25%) and phospholipids (~1%) (Yoshikawa *et al.*, 1988) and a large number of transmembrane α-helices are predicted (Lundeen *et al.*, 1987), an underestimation of α-helix content by CD spectroscopy is not unexpected. In fact, the IR data show the total amount of α-helix based on amino acid sequence has also been underestimated (Capaldi *et al.*, 1983).

Effects of CO Binding and Redox Level on CcO Secondary Structure

Second-derivative amide I spectra now appear uniquely useful for detecting subtle changes in secondary structures associated with changes in protein conformation due to minor perturbations such as changes in the oxidation state of metalloproteins (e.g., cytochrome *c*; Dong *et al.*, 1992a), the binding of ligands (Dong and Caughey, 1993), mutations (Bowler *et al.*, 1993), and partial proteolysis (A. Dong, P. Huang, B. Caughey, and W. S. Caughey, unpublished data). The superimposition of the second-derivative spectra of CcO(IV) and CcO(0) reveals changes in α-helix and turn regions that accompany the shift in redox level from fully oxidized to fully reduced $Fe_aCu_AFe_{a3}Cu_B$ redox centers (Fig. 4). Even changes in spectra as subtle as those shown in Fig. 4 are significant because they can be reliably reproduced in multiple independent experiments (Dong *et al.*, 1992a). The redox-dependent changes in turn structures suggest that movements between α-helices occur in CcO. Similar changes in the turn region of the second-derivative spectra are found upon oxygenation of hemoglobin (Dong and Caughey, 1993), a process shown by x-ray crystallography to result in large movements of α-helices (Perutz, 1970; Baldwin and Chothia, 1979). These CcO spectra also exhibit redox-dependent changes in both intensity and bandwidth for the band assigned to α-helix. However, upon binding of carbon monoxide to heme a_3 of fully reduced

CcO no changes in secondary structure could be detected in second-derivative amide I spectra. In constrast, the binding of either CO or O_2 to deoxyhemoglobin results in readily detected changes in hemoglobin amide I spectra (Dong and Caughey, 1993).

IR STUDIES OF THE INTERACTIONS OF ANESTHETICS WITH CcO

The inhibition of oxidase activity by anesthetics is of interest both in terms of the unusual nature of the inhibition and because this inhibition may contribute significantly to the anesthetic and/or toxic effects of these substances. Despite their long use, an understanding of how and where anesthetic molecules produce their various effects remains unclear (El-Maghrabi *et al.*, 1992). Both nitrous oxide and halothane have been shown to partially and reversibly reduce oxidase activity (Einarsdottir and Caughey, 1988; Dong *et al.*, 1989). The inhibition is concentration dependent up to the point of saturation where the inhibition is never greater than 50%. At clinically relevant concentrations of halothane (1 mM) and N_2O (80% N_2O, 20% O_2) inhibitions of 31 $\pm$ 5% and 27 $\pm$ 5%, respectively, have been observed (Dong *et al.*, 1989). Intriguing questions are: (1) What is the structural basis for the inhibition and (2) why is the inhibition only partial?

IR spectra of three types have provided information related to these questions. The lack of detectable perturbation of the CO IR bands of CcO(0)CO by the anesthetics shows clearly that the anesthetic molecules neither bind to nor perturb the liganded CO. Thus, anesthetics appear not to adversely affect the O_2 reduction site *per se*. The amide I spectra (Fig. 4) reveal subtle effects of the anesthetics on secondary structure. Finally, the IR spectra of the anesthetic molecules themselves can provide information on the nature of their interactions with the protein. N_2O exhibits an antisymmetric stretch band near 2230 cm^{-1} within the water window (Fig. 2). The CcO(0)CO solution minus buffer difference spectrum, both solutions saturated with N_2O, reveals a distribution of N_2O molecules among three sites of different polarity (Fig. 5). One highly polar site exhibits a band at 2235 cm^{-1}, one of intermediate polarity is at 2226 cm^{-1}, and a nonpolar site is at 2216 cm^{-1}. These assignments of site polarity are based on frequencies found for N_2O in various solvents (Gorga *et al.*, 1985; Hazzard *et al.*, 1985). The 2235 cm^{-1} site must come

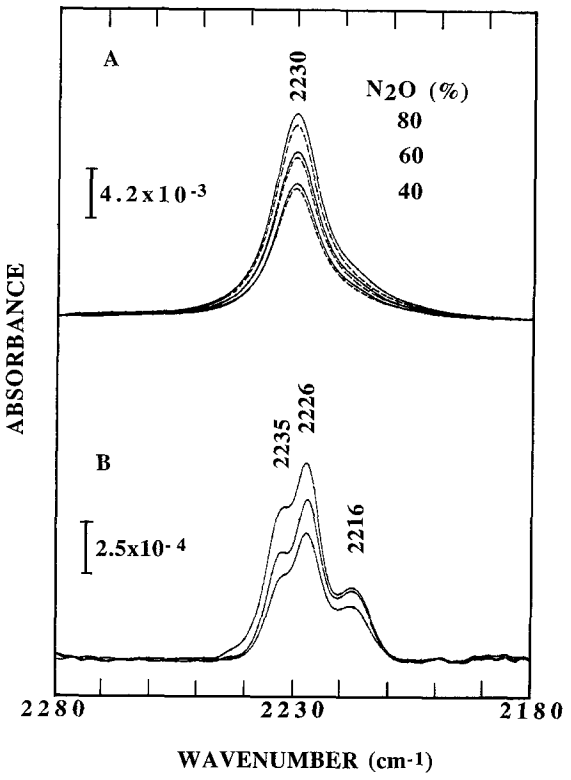

Fig. 5. Infrared spectra of N_2O in 10 mM sodium phosphate buffer and in fully reduced cytochrome *c* oxidase carbonyl solutions at 20°C. (Upper panel) Gas mixtures to which the solutions were exposed are 40% N_2O, 20% CO, 40% N_2; 60% N_2O, 20% CO, 20% N_2; and 80% N_2O, 20% CO in the oxidase solutions (——) and in the buffer (– – –). (Lower panel) difference spectra of N_2O in oxidase solutions obtained by subtracting the N_2O spectra in buffer from the N_2O spectra in oxidase solution shown in the upper panel. Three types of sites occupied by N_2O molecules within the CcO are represented by absorbances centered at 2235, 2226, and 2216 cm^{-1}.

from environments more polar than water. The 2226 cm^{-1} band represents environments analogous to acetone or dimethyl acetamide as would occur at the carbonyl of a peptide linkage. The 2216 cm^{-1} band requires a very nonpolar environment; e.g., ν_3 is 2216 cm^{-1} in cyclohexane.

These IR studies provide evidence that anesthetic molecules occupy sites within CcO that interfere with, but by no means stop, the flow of electrons from reduced cytochrome *c* to the O_2 reduction site. The structural details of this partial inhibition remain unclear. However, it is clear that anesthetic molecules represent useful probes of certain facets of CcO function and control and that IR spectra can be uniquely useful in the probing experiments.

ACKNOWLEDGMENTS

Support for the research discussed in this review from the United States Public Health Service Grant Number GM30306, the Colorado Agricultural Experiment Station Project 643, and a gift from Strohtech, Inc., is gratefully acknowledged.

REFERENCES

Alben, J. O., and Caughey, W. S. (1968). *Biochemistry* **7,** 175–183.
Babcock, G. T., and Wikstrom, M. (1992). *Nature (London)* **356,** 301–309.
Baldwin, J., and Chothia, C. (1979). *J. Mol. Biol.* **129,** 175–220.
Bazzi, M. D., and Woody, R. W. (1985). *Biophys. J.* **48,** 957–966.
Bowler, B. E., May, K., Zaragoza, T., York, P., Dong, A., and Caughey, W. S. (1993). *Biochemistry* **32,** 183–190.
Capaldi, R. A., Malatesta, F., and Darley-Usmar, V. M. (1983). *Biochim. Biophys. Acta* **726,** 135–148.
Caughey, W. S. (1980). In *Methods for Determining Metal Ion Environments in Proteins* (Darnall, D. W., and Wilkins, R. G. eds.), Elsevier, New York, pp. 95–115.
Caughey, W. S., Bayne, R. A., and McCoy, S. (1970). *J. Chem. Soc.* 950–951.
Caughey, W. S., Barlow, C. H., Maxwell, J. C., Volpe, J. A., and Wallace, W. J. (1975). *Ann. N.Y. Acad. Sci.* **244,** 1–9.
Caughey, W. S., Shimada, H., Choc, M. G., and Tucker, M. P. (1981). *Proc. Natl. Acad. Sci. USA* **78,** 2903–2907.
Chang, C. T., Wu, C.-S. C., and Yang, J. T. (1978). *Anal. Biochem.* **91,** 13–31.
Dong, A., and Caughey, W. S. (1993). *Methods Enzymol.*, in press.
Dong, A., Huang, P., and Caughey, W. S. (1989). *J. Cell. Biol.* **107,** 641a.
Dong, A., Huang, P., and Caughey, W. S. (1990). *Biochemistry* **29,** 3303–3308.
Dong, A., Huang, P., and Caughey, W. S. (1992a). *Biochemistry* **31,** 182–189.
Dong, A., Caughey, B., Caughey, W. S., Bhat, K. S., and Coe, J. E. (1992b). *Biochemistry* **31,** 9364–9370.
Einarsdottir, O., and Caughey, W. S. (1988). *J. Biol. Chem.* **263,** 9199–9205.
Einarsdottir, O., Choc, M. G., Weldon, S., and Caughey, W. S. (1988). *J. Biol. Chem.* **263,** 13641–13654.
El-Maghrabi, E. A., Eckenhoff, R. G., and Shuman, H. (1992). *Proc. Natl. Acad. Sci. USA* **89,** 4329–4332.
Fernandez Bertran, J., Pascual, J. B., and Reguera-Ruiz, E. (1990a). *Spectrochim. Acta* **46A,** 685–689.
Fernandez Bertran, J., Reguera-Ruiz, E., and Pascual, J. B. (1990b). *Spectrochim. Acta* **46A** 1679–1682.
Flamingo, F. G., Altschuld, R. A., Moh, P. P., and Alben, J. O. (1982). *J. Biol. Chem.* **257,** 1639–1650.
Gorga, J. C., Hazzard, J. H., and Caughey, W. S. (1985). *Arch. Biochem. Biophys.* **240,** 734–746.
Han, S., Ching, Y.-C., and Rousseau, D. L. (1990). *Biochemistry* **29,** 1380–1384.
Hazzard, J. H., Gorga, J. C., and Caughey, W. S. (1985). *Arch. Biochem. Biophys.* **240,** 747–756.
Hosler, J. P., Ferguson-Miller, S., Calhoun, M. W., Thomas, J. W., Hill, J., Lemieux, L., Ma, J., Georgiou, C., Fetter, J., Shapleigh, J., Tecklenburg, M. J., Babcock, G. T., and Gennis, R. B. (1993), this volume.
Krimm, S., and Bandekar, J. (1986). *Adv. Protein Chem.* **38,** 181–365.
Lundeen, M., Chance, B., and Powers, L. (1987). *Biophys. J.* **51,** 693–695.
Manning, M. C. (1989). *J. Pharm. Biomed. Anal.* **7,** 1103–1119.
Mendelsohn, R., and Mantsch, H. H. (1986). In *Progresss in Protein-Lipid Interactions*, 2nd Edn. (Watts, A., and DePont, J. J. H. M., eds.), Elsevier, Amsterdam, pp. 103–146.
Miyazawa, T., and Blout, E. R. (1961). *J. Am. Chem. Soc.* **83,** 712–719.
Myer, Y. P. (1971). *J. Biol. Chem.* **246,** 1241–1248.
Ogura, T., Takahashi, S., Shinzawa-Itoh, K., Yoshikawa, S., and Kitagawa, T. (1990). *J. Am. Chem. Soc.* **112,** 5630–5631.
Park, K., Perczel, A., and Fasman, G. D. (1992). *Protein Sci.* **1,** 1032–1049.
Perczel, A., Hollósi, M., Tusnady, G., and Fasman, G. D. (1989). *Croat. Chim. Acta* **62,** 189–200.
Perczel, A., Hollósi, M., Tusnady, G., and Fasman, G. D. (1991). *Protein Eng.* **4,** 669–679.
Perutz, M. F. (1970). *Nature (London)* **228,** 726–729.
Potter, W. T., Tucker, M. P., Houtchens, R. A., and Caughey, W. S. (1987). *Biochemistry* **26,** 4699–4707.
Potter, W. T., Hazzard, J. H., Choc, M. G., Tucker, M. P., and Caughey, W. S. (1990). *Biochemistry* **29,** 6283–6295.
Stoutland, P. O., Dyer, R. B., and Woodruff, W. H. (1992). *Science* **257,** 1913–1917.
Surerus, K. K., Oertling, W. A., Fan, C., Gurbiel, R. J., Einarsdottir, O., Antholine, W. E., Dyer, R. B., Hoffman, B. M., Woodruff, W. H., and Fee, J. A. (1992). *Proc. Natl. Acad. Sci. USA* **89,** 3195–3199.
Susi, H., and Byler, D. M. (1986). *Methods Enzymol.* **130,** 290–311.
Urry, D. W. (1972). *Biochim. Biophys. Acta* **265,** 115–168.
Urry, D. W., and Long, M. M. (1980). In *Membrane Physiology* (Andreoli, T. E., Hoffman, J. F., and Fanestil, D. D., eds.), Plenum, New York, pp. 107–124.
Varotsis, C., Woodruff, W. H., and Babcock, G. T. (1990). *J. Am. Chem. Soc.* **112,** 1297.
Yoshikawa, S., and Caughey, W. S. (1982). *J. Biol. Chem.* **257,** 412–420.
Yoshikawa, S., and Caughey, W. S. (1990). *J. Biol. Chem.* **265,** 7945–7958.
Yoshikawa, S., and Caughey, W. S. (1992). *J. Biol. Chem.* **267,** 9757–9766.
Yoshikawa, S., Choc, M. G., O'Toole, M. C., and Caughey, W. S. (1977). *J. Biol. Chem.* **252,** 5498–5508.
Yoshikawa, S., O'Keefe, D. H., and Caughey, W. S. (1985). *J. Biol. Chem.* **260,** 3518–3528.
Yoshikawa, S., Tera, T., Takahoshi, Y., Tsukikara, T., and Caughey, W. S. (1988). *Proc. Natl. Acad. Sci. USA* **85,** 1354–1358.
Yoshikawa, S., Shinzawa, K., Tsukihara, T., Abe, T., and Caughey, W. S. (1991). *J. Cryst. Growth* **110,** 247–251.
Young, L. J., and Caughey, W. S. (1986). *Biochemistry* **25,** 152–161.
Young, L. J., and Caughey, W. S. (1987). *J. Biol. Chem.* **262,** 15019–15025.