Vibrational structure of the formyl group on heme *a*

Implications on the properties of cytochrome c oxidase

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ABSTRACT Resonance Raman spectra have been recorded for heme *a* derivatives in which the oxygen atom of the formyl group has been isotopically labeled and for Schiff base derivatives of heme *a* in which the Schiff base nitrogen has been isotopically labeled. The ^{14}N–^{15}N isotope shift in the C=N stretching mode of the Schiff base is close to the theoretically predicted shift for an isolated C=N group for both the ferric and ferrous oxidation states and in both aqueous and nonaqueous solutions. In contrast, the ^{16}O–^{18}O isotope shift of the C=O stretching mode of the formyl group is significantly smaller than that predicted for an isolated C=O group and is also dependent on whether the environment is aqueous or nonaqueous. This differences between the theoretically predicted shifts and the observed shifts are attributed to coupling of the C=O stretching mode to as yet unidentified modes of the heme. The complex behavior of the C=O stretching vibration precludes the possibility of making simple interpretations of frequency shifts of this mode in cytochrome c oxidase.

INTRODUCTION

Heme *a* occurs only in cytochrome *c* oxidase (CcO), an integral membrane protein in mitochondria. CcO, as isolated, contains two such hemes, cytochrome *a* and cytochrome a_3; the former is involved in electron transfer from cytochrome *c* to cytochrome a_3 and the latter along with a copper center forms a binding site for oxygen, the physiological ligand (1). Heme *a* can be extracted from the protein and thus its structure, shown in Fig. 1, has been well defined. The formyl and farnesyl groups present in heme *a* as peripheral substituents are salient features that distinguish heme *a* from other types of heme (2). The formyl group, in particular, is a strong electron withdrawing group and its conjugation with the porphyrin macrocycle confers a unique electronic structure to the heme, which is manifested in a red-shifted visible optical absorption band (3, 4). Of greater interest is the possible role of the formyl group in proton translocation, an important biological function of CcO in vivo, via either formation of a Schiff base (5, 6) or hydrogen bonding between the formyl group and an amino acid residue of the protein (7). Being analogous to the case of rhodopsin, the former model was proposed to account for the red shift in the optical absorption maximum although spectroscopic analyses of model Schiff base complexes argue against this model (8). On the other hand, the hydrogen bonding model is based on the observation that upon reduction of the heme iron ion, there is a large downshift in the vibrational frequency of the carbonyl stretching mode of the formyl group of cytochrome *a* (7). Therefore, unambiguous identification of vibrational modes of the formyl group and its Schiff base is essential for testing these models for proton translocation.

Vibrational assignments of most of the heme *a* modes have been made indirectly by comparing the vibrational spectra of heme *a* with those of other hemes. The C=O stretching mode of the formyl group has been assigned in the 1,610–1,675 cm^{-1} region (9–11) depending on the redox and ligation states of the heme as well as on the interactions with the environment in which the heme is embedded. In the case of the protein, these interactions include steric effects, hydrogen bonding, and electrostatic forces, all of which may play an important role in the biological function of CcO. This frequency region, however, contains other vibrational modes such as the vinyl stretching mode at ~1,620 cm^{-1} (11–14), ν_{10}, a heme macrocycle mode in the 1,600–1,650 cm^{-1} range (11, 15), and the C=N stretching mode of a Schiff base (8), if any, making it somewhat difficult to assign each mode unequivocally. This region is even more complicated in the intact enzyme due to the presence of the two hemes. Therefore, to avoid these complications, it is of great value to study isolated heme *a* complexes that can be labeled with isotopes of interest.

In this paper, we report the assignments and the vibrational properties of the C=O stretching mode of the formyl group on heme *a* and C=N stretching mode of the Schiff base derivative. We describe a way of isotopically substituting the oxygen atom of the formyl

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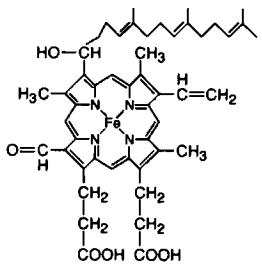

FIGURE 1 Structure of heme *a*. The important side chains include a vinyl group ($-CH{=}CH_2$) at position 4, a formyl group ($-CH{=}O$) at position 8, and a farnesyl group [$-CHOH(-CH_2CH_2CH{=}CCH_3-)_3CH_3$] at position 2.

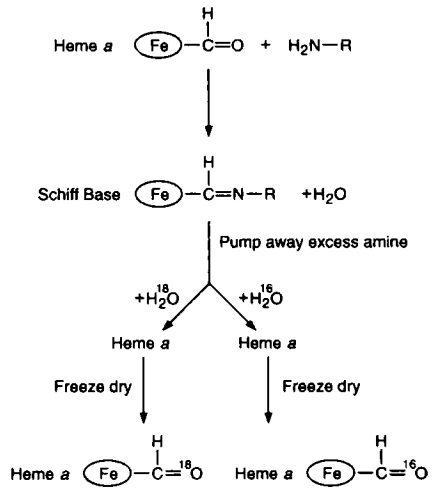

FIGURE 2 Scheme for isotopic substitution of the oxygen atom of the formyl group on heme *a*.

group of isolated heme *a* and report the ^{18}O–^{16}O shifts of the carbonyl stretching mode of the formyl group in both aqueous and nonaqueous solvents. We have also formed the Schiff base adducts of the formyl group with ^{14}N and ^{15}N in both isolated heme *a* and intact CcO and report the frequencies and the isotopic shifts of the C=N stretching modes. On the basis of these shifts we are able to show that the carbonyl group does not behave as an isolated entity. Instead, the coupling of the carbonyl stretching mode to other modes is evident. Thus, simple interpretation of frequency shifts of the vibrational modes of the formyl group must be viewed with caution.

MATERIALS AND METHODS

Heme *a* was extracted from purified CcO according to the acid-acetone method of Takamori and King (16). Optical absorption spectra were obtained on an Amino DW2000 spectrometer (SLM Instruments, Inc., Urbana, IL) using a 1-mm pathlength cell. The Raman scattering measurements were made on a previously described Raman difference apparatus (17). The laser excitation wavelength was 413 nm (Kr^+).

Extracted heme *a* (presumably a ferric chloride complex) in CH_2Cl_2 (55 μM, 250 μl) was treated with 10 μl of *n*-butylamine (^{14}N or ^{15}N) to obtain the Schiff base with two molecules of amine as axial ligands. For a Schiff base in aqueous solution, the heme *a* solution in CH_2Cl_2 was dried and resolubilized in water containing *n*-butyl amine (giving the same heme/amine relative concentrations) and 1% sodium dodecylsulfate (SDS).

Isotopic labeling of the oxygen atom on heme *a* was carried out according to the scheme shown in Fig. 2. A solution of Schiff base in CH_2Cl_2 was dried in vacuo for 1.5 h to remove excess amine and the resulting solid was dissolved in 250 μl of water (^{16}O or ^{18}O) containing 1% SDS and 0.4 M imidazole. This solution was incubated overnight at 4°C to hydrolyze the Schiff base forming a heme *a* bis-imidazole complex. The same procedure was employed in the absence of SDS and the resulting solution was freeze dried and redissolved in CH_2Cl_2 to obtain a heme *a* sample in a nonaqueous solvent. Integrity of these samples was checked by comparing their optical absorption spectra (see Figs. 3 and 4) with those of samples which had never been exposed

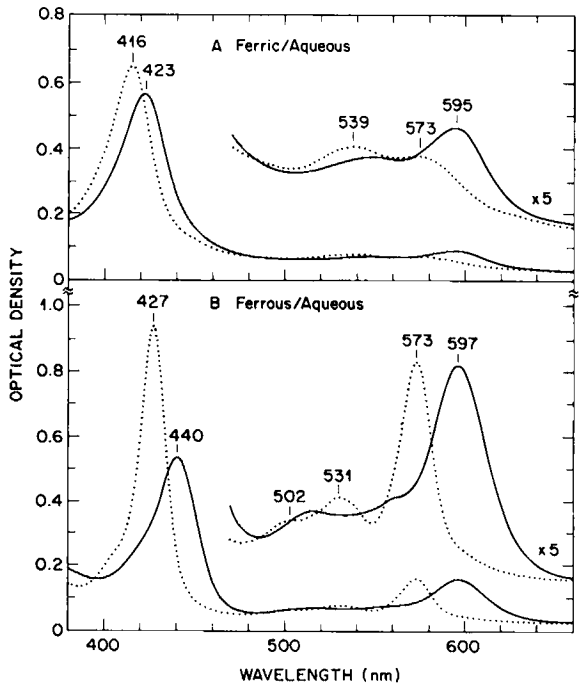

FIGURE 3 Optical absorption spectra of heme *a* (*solid line*) and its Schiff base derivative (*dashed line*) in aqueous solution for both the ferric (*top*) and ferrous (*bottom*) oxidation states. The Schiff base was formed by adding *n*-butylamine to heme *a*. In both cases the heme was six-coordinate low spin with the axial ligands of imidazole for heme *a* and *n*-butylamine for the Schiff base. The heme concentration was 55 μM and the pathlength of the absorbance cell was 0.1 cm.

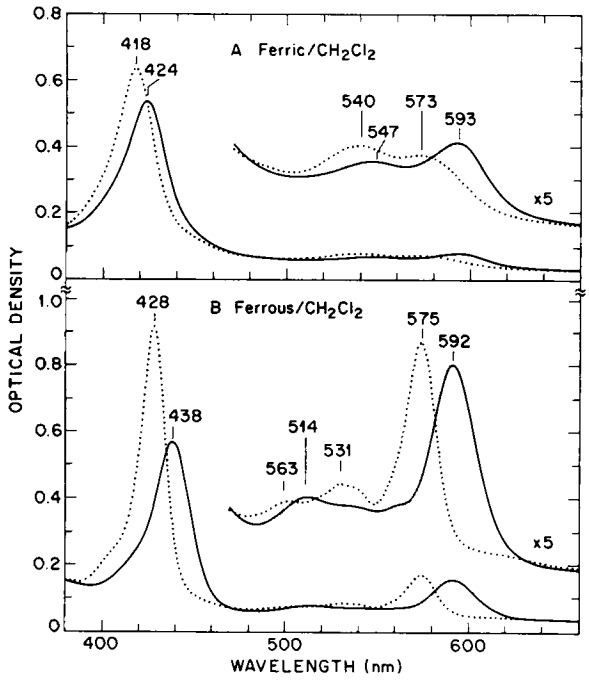

FIGURE 4 Optical absorption spectra of heme *a* (*solid line*) and its Schiff base derivative (*dashed line*) in nonaqueous solutions for both the ferric (*top*) and the ferrous (*bottom*) oxidation states. The conditions are the same as those in Fig. 3.

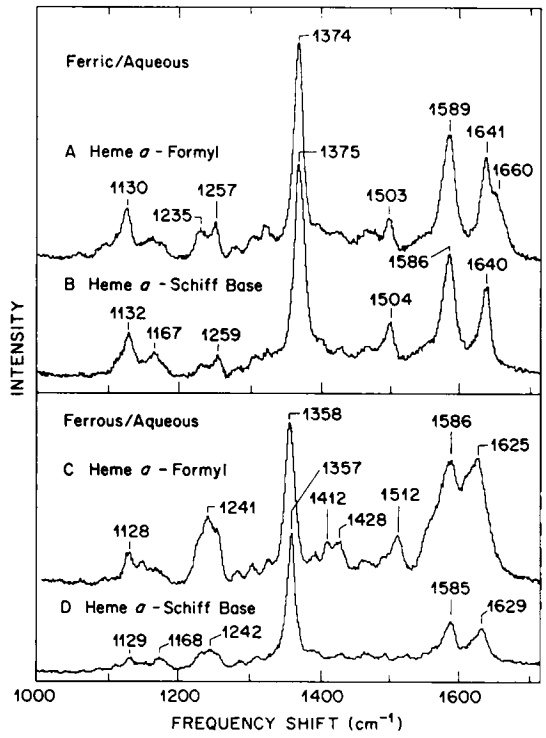

FIGURE 5 Resonance Raman spectra in the high frequency region of heme *a* and is Schiff base derivatives in aqueous solution in the ferric (*A*, heme *a*; *B*, the Schiff base derivative) and the ferrous (*C*, heme *a*, and *D*, the Schiff base derivative) oxidation states. The conditions are the same as those for the samples used for the optical absorption spectra reported in Fig. 3. All the spectra were excited at 413.1 nm.

to amine. Ferric complexes of heme *a* and its Schiff base derivative were reduced by a small excess of sodium dithionite dissolved in water (aqueous samples) or in crown ether/methanol (nonaqueous samples).

RESULTS

The formyl group of heme *a* undergoes a condensation reaction with various amines to form a Schiff base. The reaction is reversible depending on the concentration of the reactive (unprotonated) amine in the solution. We found that ferrous heme *a* requires substantially higher amine concentration to complete the Schiff base formation than the ferric form, indicating that the equilibrium between heme *a* and its Schiff base depends on the redox state of the iron atom. The equilibrium can also be pushed toward formation of the formyl group on heme *a* by lowering the pH of the solution, making the amine unreactive due to its protonation.

The resonance Raman spectra of six coordinate heme *a* in aqueous solution are shown in Fig. 5. The heme spin marker line (ν_2) in the 1,550–1,590 cm^{-1} region (11, 15) confirms that the iron atom is in its low spin configuration resulting from binding two imidazole molecules at the fifth and sixth positions on the iron atom. If the iron were five coordinated, a high spin configuration would be expected. Formation of a Schiff base with *n*-butylamine, as described in the previous section, yields spectra which are nearly identical to those of the heme with the formyl constituent. The only large change occurs in the high frequency region (Fig. 5) where the C=O stretching mode of the formyl group and the C=N stretching mode of the Schiff base are expected. Hydrolysis of the Schiff base regenerates heme *a* with the formyl side chain. This was confirmed by the optical absorption and the resonance Raman spectra which were identical to the original samples before formation of the Schiff base (data not shown).

A. Isotopically substituted formyl groups

To obtain a clear assignment of the C=O stretching mode of the formyl group we have hydrolyzed the Schiff base in $H_2{}^{16}O$ and $H_2{}^{18}O$, thereby isotopically substitut-

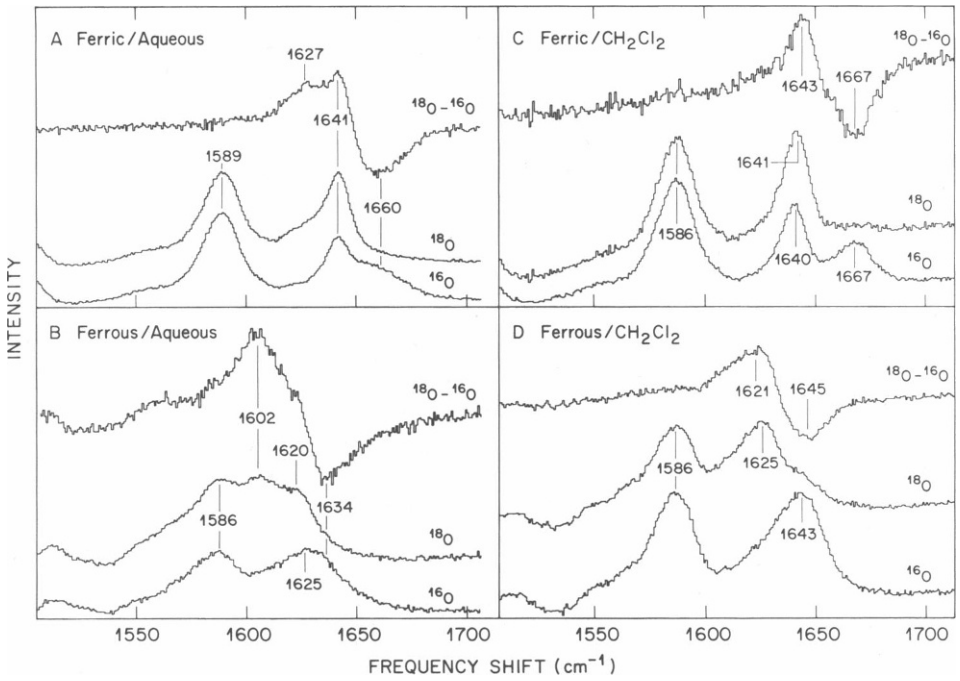

FIGURE 6 Resonance Raman spectra and associated difference spectra in the regin of the C=O stretching mode of the formyl group of heme *a* comparing C=^{16}O to C=^{18}O. (*A*) Ferric heme in aqueous solution. (*B*) Ferrous heme in aqueous solution. (*C*) Ferric heme in CH_2Cl_2. (*D*) Ferrous heme in CH_2Cl_2. The heme (concentration, 55 μM) is six-coordinate with imidazole axial ligands.

ing the oxygen atom on the formyl group. The spectra in the region of the C=O stretching mode of the formyl group for both the ferrous and ferric oxidation states of the heme in aqueous and organic solvents are shown in Fig. 6. These frequencies and the isotopic shifts were determined both by the difference spectra, shown in Fig. 6, and by deconvoluting the spectra with a curve fitting procedure. The results are summarized in Table 1. Several additional observations are evident from the data in Fig. 6: (*a*) The isotopic shifts of the C=O stretching mode are clear and straightforward for all forms of heme *a* that we studied except ferric heme *a* in an aqueous environment. (*b*) The oxygen isotope shifts are about 33 cm^{-1} in aqueous solution and 20–24 cm^{-1} in nonaqueous solution. (*c*) Within a given oxidation state, the carbonyl stretching mode of the formyl group shifts to higher frequency by ~8cm^{-1} in changing from aqueous to nonaqueous solution. (*d*) Within the same environment (aqueous or nonaqueous), the carbonyl stretching mode shifts to lower frequency by ~25 cm^{-1} when the oxidation state of the iron changes from ferric to ferrous.

TABLE 1 Frequencies and isotopic shifts (cm) of C=O stretching mode of the formyl group and the C=N stretching mode of the Schiff base derivatives of six-coordinate low spin heme *a*

Heme *a*	Solvent	$\nu_{C=O}$	$\Delta\nu_{^{16}O-^{18}O}$	$\nu_{C=N}$	$\Delta\nu_{^{14}N-^{15}N}$
Ferric	CH_2Cl_2	1,667	24	1,640	20
Ferric	Aqueous	1,660	33	1,643	25
Ferrous	CH_2Cl_2	1,644	20	1,632	18
Ferrous	Aqueous	1,635	33	1,632	22

B. Schiff base derivatives

Isotopic substitution studies of the Schiff base were also carried out by reacting the heme with ^{15}N-labeled *n*-butyl-amine. A nitrogen isotope–sensitive line was detected at ~1,640 cm^{-1} in the ferric forms of the heme and at 1,632 cm^{-1} in the ferrous forms. Based on the isotope shift and the observations of similar frequencies from the C=N entity in other systems, we assign this line as the C=N stretching mode of the Schiff base derivative of heme *a*. In general, the spectra were very similar to those of heme *a* with the formyl side chain except in the region of the C=O and C=N stretching modes. Thus, forming these derivatives does not affect the oxidation state or the axial coordination. The results of the isotope studies are shown in Fig. 7 and listed in

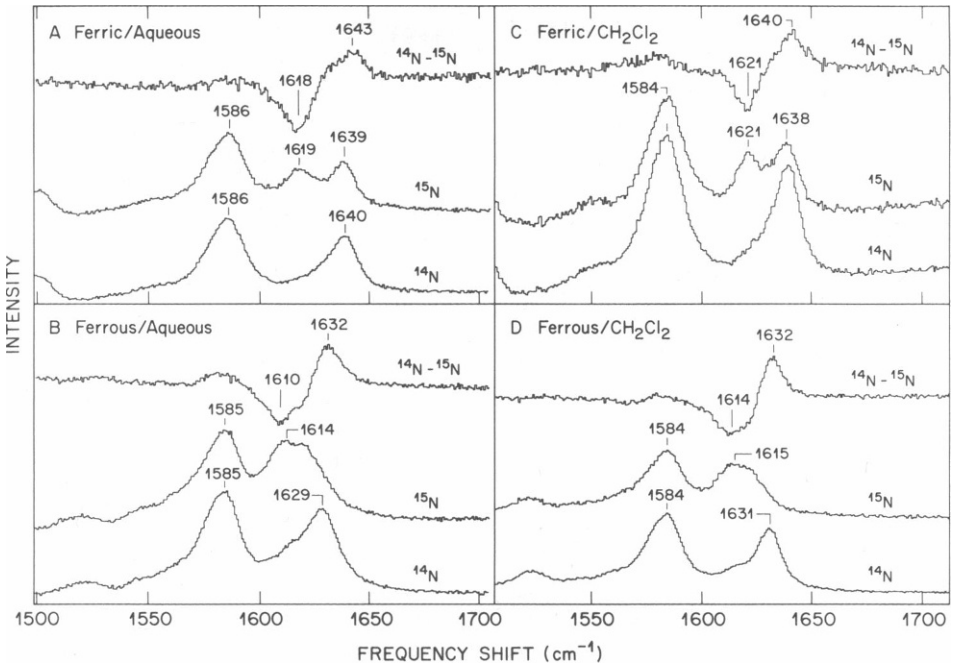

FIGURE 7 Resonance Raman spectra and associated difference spectra of Schiff base derivatives of heme *a* in the region of the C=N stretching mode of the Schiff base comparing C=^{14}N to C=^{15}N. (*A*) Ferric derivative in aqueous solution. (*B*) Ferrous derivative in aqueous solution. (*C*) Ferric derivative in CH_2Cl_2. (*D*) Ferrous derivative in CH_2Cl_2. The heme (concentration 55 μM) is six-coordinate with *n*-butylamine axial ligands.

Table 1. Several; features are evident from these data: (*a*) The isotopic shifts are clear and straightforward for all forms of the Schiff base which we examined and in the range of 18–25 cm^{-1}. There is no evidence for strong interactions with any other heme modes. (*b*) The frequency of the C=N stretching mode of the Schiff base does not shift significantly upon changing from an aqueous to a nonaqueous environment. (*c*) The C=N stretching mode shifts to lower frequency by ~10 cm^{-1} upon changing from the ferric to the ferrous form of the heme.

To determine the effect of forming a Schiff base in the intact protein, we have reacted cytochrome *c* oxidase with amines as well. The resulting spectra of the ferric and ferrous forms of the protein are illustrated in Fig. 8. Isotope shifts of the same magnitude (17–20 cm^{-1}) as those in the model compounds were detected. Attempts to hydrolyze the Schiff bases to reconstitute the original enzyme were unsuccessful. Because the raw spectra contain contributions from the C=N stretching mode of the Schiff base in addition to contributions from heme modes such as ν_{10}, we have utilized the isotope difference spectra to determine the frequency of the Schiff base modes. The values found for the C=^{14}N stretching mode are 1,640 and 1,631 cm^{-1} for the ferric and ferrous heme oxidation states, respectively.

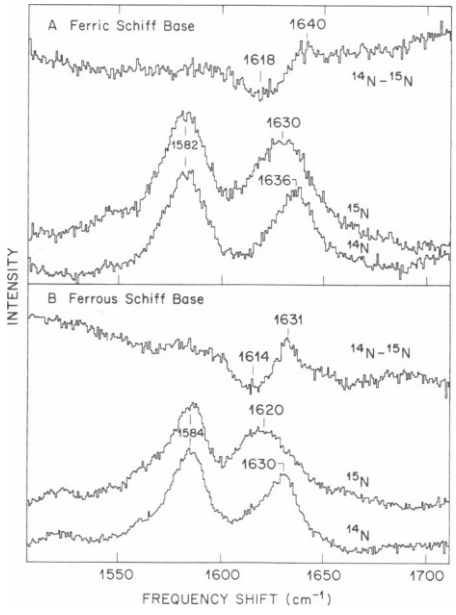

FIGURE 8 Resonance Raman spectra and associated difference spectra in the region of the C=N stretching mode of the Schiff base derivatives of cytochrome *c* oxidase formed by the addition of *n*-butylamine to the enzyme. The comparison between C=^{14}N and C=^{15}N allows for identification of the stretching mode in the intact enzyme. The enzyme concentration was 55 μM.

DISCUSSION

A. Formyl group of heme *a*

The hemes (heme *a*) in CcO have a formyl group substituent but its functional role has not been determined. The vibrational frequencies of the carbonyl stretching mode of the formyl groups in the enzyme have been assigned in the past (9–11) and illustrate that the formyl group of cytochrome *a* behaves very differently from that of cytochrome a_3. Specifically, the C=O stretching frequencies of the ferric and ferrous forms of cytochrome *a* are located at 1,650 and 1,610 cm^{-1}, respectively, whereas the corresponding frequencies in cytochrome a_3 are located at 1,676 and 1,665 cm^{-1}, respectively. (An additional frequency of 1,644 cm^{-1} for a low-spin ferrous form of cytochrome a_3 has also been reported [18].) The large differences between cytochrome *a* and cytochrome a_3 were attributed by Babcock and Callahan (7, 11) to a difference in the strength of hydrogen bonding to the formyl groups. The interactions which give rise to the frequency differences is of utmost importance because the strong hydrogen bonding that was inferred has been proposed to play an essential role in proton translocation (7).

To study the vibrational properties of the formyl group on heme *a* we have generated oxygen-18 isotopic derivatives of heme *a* by forming a Schiff base and then hydrolyzing it in $H_2{}^{18}O$. The only similar isotopic substitution studies which have been reported were done on a copper porphyrin derivative containing a formyl and a vinyl group (19). In the present study we have been able to determine the effect of the iron oxidation state and the effect of an aqueous versus a nonaqueous environment on the properties of the formyl group by identifying both the frequencies and the oxygen isotopic shifts.

To determine the frequencies of the C=O stretching mode of the formyl group we have utilized a deconvolution procedure based on Marquardt's algorithm (20–22) and Raman difference spectroscopy of the isotopically substituted preparations. This has allowed very accurate determination of the frequencies even in those cases in which the carbonyl stretching mode is overlapped by other modes. From this analysis, the difference in the frequencies of the C=O stretching modes of the formyl group on the six-coordinates low spin heme *a* between an aqueous and a nonaqueous heme environment for both the ferric and the ferrous oxidation states was found to be ~ 8 cm^{-1}. This difference and the absolute frequencies that we obtain are in agreement with those reported for ferrous heme *a* model compounds by others (11, 15, 19, 23). In the nonaqueous solutions our frequency of 1,644 cm^{-1} compares with reported frequencies of 1,642–1,645 cm^{-1}; and our frequency in aqueous solution of 1,635 cm^{-1} compares to reported values ranging from 1,623–1,635 cm^{-1}. However, for the ferric case the difference in the frequency of the formyl mode between aqueous and nonaqueous solutions is somewhat smaller than that which some others have reported (11, 19) but close to those reported by Choi et al. (15). This results from our identifying the carbonyl stretching mode (1,667 cm^{-1}) at a somewhat lower frequency than others (11, 19) have reported (1,670–1,673 cm^{-1}) in nonhydrogen bonding solvents and at a slightly higher frequency than those of others (1,655–1,656 cm^{-1}) (11, 19) in aqueous solutions. (Our values of 1,660 and 1,667 cm^{-1} in aqueous and nonaqueous solvents, respectively, compare best with those of Choi et al. (15) at 1,656 and 1,666 cm^{-1}.) We believe that the differences between our frequencies and those of others results from our ability to be able to better identify the center frequencies by using the oxygen isotope differences. However, it must be pointed out that still lower frequencies were found by Centeno et al. (19), when the heme was placed in solvents with greater proton donating power than H_2O (1,650 and 1,626 cm^{-1} for the ferric and ferrous cases, respectively).

In CcO, the difference in the carbonyl stretching frequency between the ferric forms of cytochrome *a* and cytochrome a_3 is ~ 20 cm^{-1} and the corresponding difference in the ferrous forms is > 50 cm^{-1} (11). Thus, the small differences which we detect between an aqueous and a nonaqueous environment of 8 cm^{-1} cannot account for the large differences of the carbonyl vibrational frequencies of the heme formyl groups in the protein. The additional shift (19) realized by placing the heme in a solvent with a lower pK_a than H_2O is at most 10 cm^{-1} so the large differences cannot be attributed exclusively to strong hydrogen bonding. Other interactions between the formyl group of the heme and the protein must contribute to the difference between cytochrome *a* and cytochrome a_3. This conclusion is supported by the results of Tsubaki et al. (24) who examined myoglobins reconstituted with hemes in which formyl groups were substituted for one or both vinyl groups on heme *b*. For the three possible substitutions (2F-2V, 2V-4F, and 2F-4F) they found that the C=O stretching frequencies were 1,648, 1,660, and 1,668 cm^{-1}, respectively. These frequency difference could be attributed to different degrees of π-donation from the formyl groups into the heme, and not to different degrees of hydrogen bonding for the two positions on the heme. In that study, high spin to low spin conversion of the substituted ferric forms of the myoglobin had no effect on the vibrational frequency of the formyl group.

A factor which can influence the frequency displayed by a given vibrational mode is the strength of coupling of that mode with other modes of the system. Such cou-

pling often becomes evident by comparing the calculated isotopic shift for an isolated oscillator to the observed shift. For an isolated C=O stretching mode on a formyl group at 1,650 cm^{-1}, the calculated ^{16}O–^{18}O frequency shift is 40 cm^{-1}. The data in Table 1 demonstrate that all of the oxygen isotope shifts are substantially smaller than the theoretical shift. In the nonaqueous environment the shift is 20–24 cm^{-1} and in aqueous solution it is 33 cm^{-1}. Thus, it becomes clear that the carbonyl stretching mode is highly coupled to other modes. From the data we are unable to determine which modes are involved in the coupling for all cases except for the ferric heme in an aqueous environment. In that case the isotopic shift shows involvement with a mode at 1,627 cm^{-1}, possibly ν_{10} or the vinyl stretching mode. In their study of myoglobins reconstituted with formyl substituted heme *b*, Tsubaki et al. (24) suggested that a heme combination mode couples with the carbonyl mode of the formyl group. In examining the observed Raman spectra of heme *a*, clear candidates for a combination mode which could couple to the carbonyl mode are not evident. However, the isotope shifts confirm that the normal coordinate cannot be described by an isolated C=O stretching mode. Furthermore, the large difference in the isotope shift, dependent on whether the solvent is aqueous or nonaqueous, indicates that the normal coordinate is sensitive to the environment. Thus, it is difficult to draw simple conclusions concerning the structure and bonding of the formyl group based on frequencies without a full understanding of the local environment.

B. Schiff base derivatives

The general behavior of the Schiff base derivatives is quite different from that of the formyl derivatives. For example, it was observed that in both the ferric and the ferrous oxidation states of the heme, the C=N stretching frquencies were independent of whether the environment was aqueous or nonaqueous. This is also evident in the optical absorption spectra (Figs. 3 and 4) which demonstrate that the solvent has a larger effect on the position of the alpha band for native heme *a* than for the Schiff base derivative. Thus, unlike the formyl group, there does not appear to be a large interaction between the Schiff base and its local environment within the limitations of our experiments. Based on the observation that the C=N stretching frequency is the same in the aqueous environment as it is in the nonaqueous environment, we also conclude that the Schiff base is not protonated in either of these cases. Further evidence is provided by the fact that the C=N stretching modes show no shifts when incubated in D_2O. Attempts to form a protonated Schiff base by the addition of strong proton donors were not successful but are still under investigation.

The theoretical isotope shift for an isolated C=N group with a frequency of 1,640 cm^{-1} is 25 cm^{-1}. The frequency shifts which we have detected are in reasonable agreement with this predicted value with a range of 18–25 cm^{-1}. Thus, the coupling of the C=N vibration with other modes of the heme appears minimal in contrast to the larger coupling detected for the formyl group. The electronic coupling of the C=N to the heme is similar to that of the formyl group as evidenced by the observation that each have similar shifts to lower frequency upon changing the oxidation state from the ferric to the ferrous form.

The ^{14}N–^{15}N isotope shift found when a Schiff base is generated in CcO is the same as that which we detect in the isolated model heme. That both hemes (cytochrome *a* and cytochrome a_3) form Schiff bases under our conditions is confirmed by the absence of any remaining formyl lines in the spectrum. However, although we have no evidence to the contrary, we cannot say with certainty that both hemes have formed the Schiff base adduct with the added *n*-butylamine and not with an amino acid residue of the protein due to the high pH conditions resulting from the presence of the added amine. It has been reported that, at a high pH, Schiff base formation between the formyl group of heme *a* and amino acid residues can take place (4, 10).

CONCLUSIONS

The results reported here firmly establish the identification of the carbonyl stretching modes of the formyl group of heme *a* and the C=N stretching mode of the Schiff base derivative of heme *a* for both oxidation states of the iron atom. Solvent effects on and isotope shifts of the C=O stretching mode demonstrate that the C=O group cannot be described as an isolated unit. Instead, the coupling of the C=O stretching motion to other degrees of freedom of the heme and possibly the solvent is influenced by the environment. Such coupling is not evident in the C=N stretching mode of the Schiff base derivative. Additional experiments are needed to further clarify the nature of the coupling and the specific modes or combination of modes that are involved in the coupling.

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