Articles

Structure of Cytochrome b_5 in Solution by Fourier-Transform Infrared Spectroscopy†

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ABSTRACT: Fourier-transform infrared spectroscopy was used to examine the secondary structure of rabbit liver cytochrome b_5 and the polar and nonpolar domains of the protein. The data for both the polar and nonpolar domains agree well with those previously obtained by other physical techniques. In particular it was found that the nonpolar membrane-binding domain was predominantly α helix and that the polar domain was also highly helical, but not all α helix. The independence of the two domains in the whole molecule was, in general, confirmed by the additivity of the spectra of the two domains. The small differences that were seen indicate that there is a loss of α helix when the protein is cut into the two domains. In addition, there appeared to be a slight difference in the exposure to solvent of the amide NH groups in the α-helical portion of the nonpolar domain when it was examined in isolation.

Cytochrome b_5, an amphipathic integral membrane protein found in the endoplasmic reticulum, plays an important role in lipid metabolism (Holloway, 1983) and, because of its relative ease of purification (Ozols, 1974), has been a popular subject for model membrane studies. Several studies have probed the topography of the protein when it is bound to lipid vesicles via its nonpolar membrane-binding domain (Flemming et al., 1979; Takagaki et al., 1983; Gogol & Engelman, 1984; Markello et al., 1985, Kleinfeld & Lukacovic, 1985; Everett et al., 1986; Rzepecki et al., 1986; Arinc et al., 1987) but a complete interpretation of these data is hampered by the lack of information on the secondary structure of this domain. The polar heme-containing domain has been crystallized and subjected to X-ray analysis (Mathews et al., 1971), but the whole native protein, and the nonpolar domain in particular, have not yet been crystallized. Structures have been predicted for the nonpolar domain based on Chou and Fasman calculations and circular dichroism measurements (Visser et al., 1975; Dailey & Strittmatter, 1978; Tajima et al., 1978; Hlavica, 1984), but it was felt that the recent advances in FT-IR spectroscopy would be able to give an alternate approach to the determination of the secondary structure of the nonpolar domain. The results in this paper are in good agreement with the circular dichroism measurements.

Materials and Methods

Cytochrome b_5 was prepared as described previously (Markello et al., 1985). The protein was cleaved with TPCK-trypsin (in a ratio of 1 trypsin/10 cytochrome molecules) in 10 mM Tris–acetate, pH 8.1, containing 10 mM $CaCl_2$. The mixture was kept at 4 °C overnight, and the cloudy mixture was centrifuged at 10000*g* for 10 min. UV spectral analysis indicated almost complete retention of the polar domain in the supernatant with the nonpolar domain in the precipitate. The polar domain was purified further by chromatography on DEAE-cellulose with a linear gradient from 50 to 200 mM potassium phosphate buffer (pH 7.2). The nonpolar domain was dissolved in glacial acetic acid and subjected to gel filtration on Sephadex G-75 in 50% acetic acid–water. The peptide-containing fractions, which eluted just before the green heme band, were pooled and lyophilized. All proteins and peptides gave single bands on gel electrophoresis. Before FT-IR analysis, all samples were subjected to gel filtration on Sephadex G-25 in 10 mM NH_4HCO_3 to remove acetate ions (which produce an interfering infrared band at 1560 cm^{-1}) and lyophilized.

Infrared spectra were recorded at 22 °C with a Digilab FTS-60 instrument using a high-sensitivity DTGS detector. For each spectrum 256 interferograms were collected, coadded, apodized with a Bessel function, and Fourier transformed to give a resolution of 2 cm^{-1}. Samples were prepared in 50 mM HEPES buffer in D_2O (pD 8.0) at protein concentrations between 1 and 2 mM and were assembled between CaF_2 windows separated with a 50-μm Teflon spacer. For spectra in H_2O a 6-μm spacer was used. Fourier self-deconvolution was performed by using a Lorentzian of 25-cm^{-1} half-bandwidth and a resolution enhancement factor (k value) of 2.8.

Results and Discussion

Polar Domain. The spectra of the polar domain in H_2O and D_2O are shown in Figure 1A. Infrared spectra of proteins in H_2O typically show two broad bands, one between 1700 and 1600 cm^{-1} and one between 1600 and 1500 cm^{-1}. The former band is the amide I band, and the latter is a complex of the amide II band and bands due to side-chain vibrations. Both amide bands are complex composites of several discrete bands that are characteristic of specific types of secondary structure in the protein. In D_2O the accessible N–H groups will undergo H → D exchange and there will be a small shift of the component bands of the broad amide I band and a large shift, down to below 1500 cm^{-1}, of the amide II band. As seen in Figure

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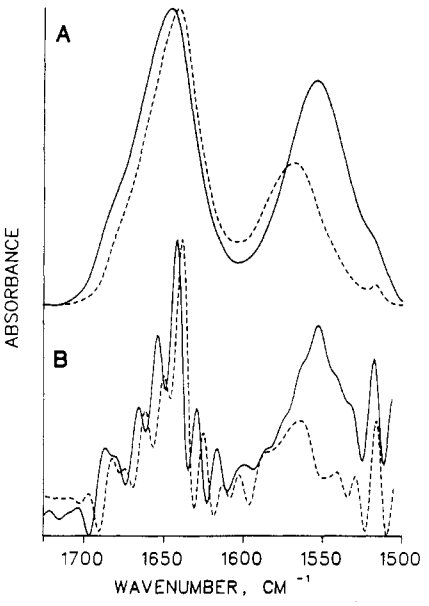

FIGURE 1: Infrared spectra of the polar domain of cytochrome b_5 in H_2O and D_2O in the region of the amide I and amide II bands (1700–1500 cm^{-1}). FT-IR spectra of the polar domain were obtained as described under Materials and Methods. In (A) are shown the original infrared spectra and in (B) the spectra after band enhancement by Fourier self-deconvolution (Mantsch et al., 1986). (—) Sample in H_2O buffer; (- - -) sample in D_2O buffer.

1A, the shift of the 1650-cm^{-1} band between the two solvents indicates that extensive exchange of H for D has occurred. This is supported by the large observed decrease in intensity of the amide II band centered at around 1550 cm^{-1}. The large peak that still remains in the spectrum in D_2O, at 1570 cm^{-1}, is contributed to by the 21 mol % of Asp and Glu that the polar domain contains.

More information can be obtained from the rather featureless amide I bands by a process of Fourier self-deconvolution (Mantsch et al., 1986), as was performed to produce the curves in Figure 1B, where it can be seen that each of the component bands of the amide I band is shifted by approximately 5 cm^{-1} to lower frequencies in D_2O but with the relative intensities of the bands maintained. This observed shift had occurred within 1 h, and there was no further shift with longer times. It was of some concern that the Fourier self-deconvolution of the spectra in H_2O may produce artifactual peaks, depending on the accuracy of the subtraction of the very large H_2O band centered at 1640 cm^{-1}. However, it was found that deliberate over- or undersubtraction of the H_2O peak did not alter either the positions or relative intensities of the peaks in the deconvolved spectra.

In the deconvolved spectrum of the D_2O sample (Figure 1B) it can also be seen that the actual absorbance at 1550 cm^{-1}, due to the amide II band, is very small, again confirming that there has been extensive H → D exchange. In this spectrum can also be seen other bands that are due to specific vibrations of the amino acid side chains (Chirgadze et al., 1975; Surewicz & Mantsch, 1988; Arrondo et al., 1988). Most notable are those of the Tyr at 1614.5 and 1516.5 cm^{-1}, which are shifted from their positions seen in the spectrum in H_2O, and the overlapping bands due to the antisymmetric stretching mode of the COO^- of the 11 Glu at 1564 cm^{-1} and the 8 Asp at 1585 cm^{-1}.

The quantitative contribution of each band to the total amide I contour may be obtained by curve-fitting procedures. This curve fitting can be done either with the original spectra or with the deconvolved curves, as it has been shown that, with a good signal-to-noise ratio, the areas of the bands in the deconvolved spectra are an accurate assessment of the band intensities of the original amide I band (Mantsch et al., 1988). Two examples of the curve fitting to the deconvolved spectra for the polar domain in the two solvents are shown in Figure 2. In Figure 2A the six bands between 1700 and 1625 cm^{-1} are considered to be due to the vibrations of the peptide bond; the one band shown below 1625 cm^{-1}, although included in the curve-fitting procedure, is due to side-chain vibrations. In Figure 2B there are again six bands to be considered; the one at approximately 1612 cm^{-1} is again due to amino acid side chains. The assignment of these bands is based on previous studies of proteins by vibrational spectroscopy and is somewhat simplified in this case by the exact duplication, with the aforementioned 5-cm^{-1} shift, of the spectral bands in H_2O versus D_2O. This shift obviates the need for any discussion of partial deuterium exchange in the band assignments; complete exchange must have occurred. It should be pointed out that the similarity of the profiles in parts A and B of Figure 2, sample in H_2O versus sample in D_2O, provides more evidence that there have been no artifacts introduced by buffer subtraction and deconvolution. The visual duplication seen in Figure 1B is confirmed by the data in Table I. The percentage of intensity in each band seen in H_2O is well matched by the intensity of the band seen in the D_2O spectrum, shifted by approximately 5 cm^{-1}. The assignments of the bands in D_2O can be summarized as follows: the bands at 1683, 1675, and 1625 cm^{-1} are due to β structures; the band at 1650 cm^{-1} is due to α helices; and the band at 1662 cm^{-1} is due to turns.

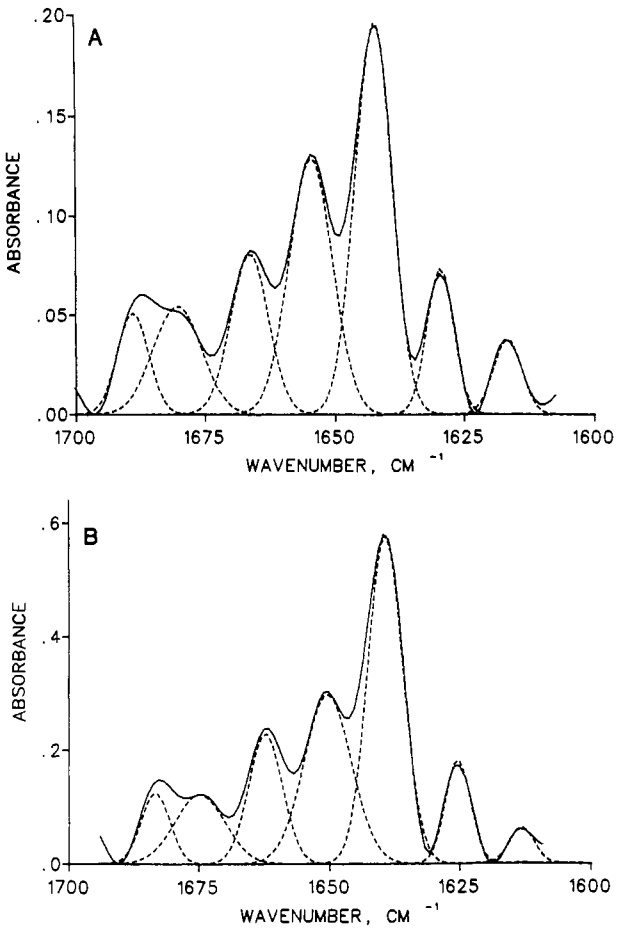

FIGURE 2: Curve fitting to the deconvolved spectra of the polar domain of cytochrome b_5 in the region of the amide I band (1700–1600 cm^{-1}). The infrared spectra were obtained as described for Figure 1 and were subjected to a standard curve-fitting procedure. (A) Sample in H_2O; (B) sample in D_2O.

Table I: Positions and Fractional Areas of the Amide I Bands of Cytochrome b_5 and Its Two Domains[a]

	H_2O		D_2O		
protein	band position (cm^{-1})	band area (%)	band position (cm^{-1})	band area (%)	assignment
polar	1689	7	1683	6	β
domain	1680	12	1675	11	β
	1667	14	1662	14	turns
	1655	25	1650	25	α
	1643	33	1639	35	3_{10}
	1630	9	1625	9	β
nonpolar	1678	19	1677	10	β
domain			1663	12	turns
	1657	54	1655	43	α
			1645	12	random
	1641	14	1636	19	β
	1630	13	1628	4	β
whole	1686	6	1683	3	β
cytochrome	1680	10	1675	8	β
b_5	1667	13	1662	15	turns
	1656	46	1651	33	α
	1642	18	1639	33	$3_{10} + \beta$ + random
	1629	7	1625	8	β
sum of	1690	5	1683	4	
polar plus	1680	11	1675	11	
nonpolar	1667	10	1662	15	
domains[b]	1656	40	1652	30	
	1642	23	1639	33	
	1630	11	1626	7	

[a] The infrared spectra, after Fourier self-deconvolution, were subjected to curve fitting, and the percentages shown are the relative areas of the bands that make up the amide I band. The error in these assignments is estimated to be quite small. The signal-to-noise ratio of better than 10000:1 for the original spectra was degraded to approximately 200:1 by the Fourier self-deconvolution procedure. The reproducibility of the curve-fitting procedure depended upon the particular domain being examined and was worse for the nonpolar domain. With the nonpolar domain, three independent curve-fitting trials produced values of 44 ± 2%, 30 ± 3%, 13 ± 1%, and 14 ± 3% for α helix, β structure, turns, and random structure, respectively. [b] This is an analysis of a composite spectrum produced by a weighted addition of the spectra of the polar and nonpolar domains. The composite spectrum was subjected to Fourier self-deconvolution and curve fitting as described under Materials and Methods.

The major band in the deconvolved spectrum in D_2O, at 1639 cm^{-1}, offers a more difficult assignment. It is at a frequency that has been assigned to random structure and β structure, although 1639 cm^{-1} is low for random structure and high for β structure. In the case of this polar domain of cytochrome b_5, an additional possibility is 3_{10} helix. X-ray analysis of cytochrome b_5 has shown a preponderance of this latter structure, and the assignment of the corresponding infrared band is not known. We suggest that the band at 1639 cm^{-1} seen in this spectrum is due to 3_{10} helix. The general features of the polar domain from these first FT-IR studies are (see Table I) α helix 25%, turns 14%, 3_{10} helix 35%, and β structure 26%.

Previous comparisons of X-ray and circular dichroism measurements have discussed the discrepancies between these two methods of structure determination (Huntley & Strittmatter, 1972). Mathews et al. (1971) concluded from their analysis of the crystal structure at 2-Å resolution that 50% of the amino acid residues were in helices and 25% in β structure. They did note that none of the six short helices had complete α-helical geometry, at most they had 1.5 turns of α helix with the rest being 3_{10}. They also noted that "there are several bifurcated hydrogen bonds in the helical regions and occasional regions where several NH groups share several

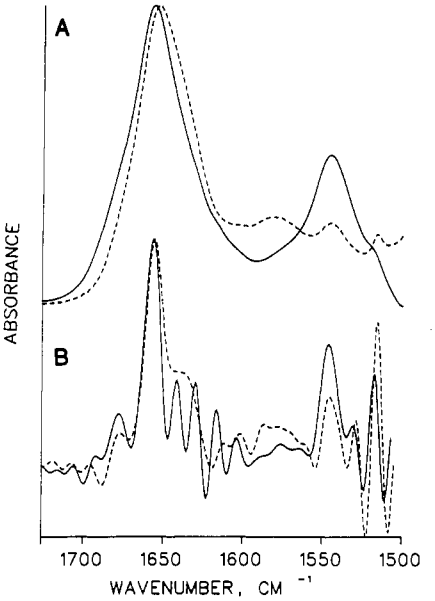

FIGURE 3: Infrared spectra of the nonpolar domain of cytochrome b_5 in H_2O and D_2O in the region of the amide I and amide II bands (1700–1500 cm^{-1}). FT-IR spectra of the nonpolar domain were obtained as described under Materials and Methods. In (A) are shown the original infrared spectra and in (B) the spectra after band enhancement by Fourier self-deconvolution. (—) Sample in H_2O buffer; (- - -) sample in D_2O buffer.

carbonyl oxygen atoms". Huntley and Strittmatter (1972) suggested that the values calculated by them, 19% α helix, 36% β structure, and 45% random structure, probably overestimate the β structure and underestimate the helical structure (α plus 3_{10}) because of problems of calculating the amount of 3_{10} helix from circular dichroism data. The unusual nature of the helical domains would also explain the low α-helical content found by FT-IR, although, if our suggested assignment of the band at 1639 cm^{-1} is correct, the total helical content determined by FT-IR (60%) is closer to that seen by X-ray analysis (50%). In rationalizing the assignment of the 1639-cm^{-1} band to 3_{10} helix, it should be noted that the latter helix is a tighter helix. Such bonds, especially with the bifurcations seen by Mathews et al. (1971), would be expected to show bands at a frequency lower than those of α helices. These same comments may explain the completeness of the H → D exchange demonstrated in Figure 1B. Also, the polar domain of cytochrome b_5 is a small protein, only 89 amino acids, and the regions of rather distorted helix would certainly be more prone to H → D exchange than would a normal α helix.

Nonpolar Domain. The spectra of the hydrophobic nonpolar domain in D_2O and H_2O are shown in Figure 3. Again, it is apparent that there is a shift of the amide I band to lower frequencies in D_2O and a decrease in the intensity of the amide II band in the D_2O spectrum. The changes in both of these bands indicate some H → D exchange has occurred, although the amount of exchange is less than that observed with the polar domain. This can be most readily seen in the deconvolved spectra in Figure 3B and in Table I, where it is apparent that the major band at 1657 cm^{-1} only shifts to 1655 cm^{-1}. In addition, there is a much larger residual amide II band at 1545 cm^{-1} in D_2O with the nonpolar domain than was seen with the polar domain (compare Figures 1B and 3B). Another major difference between the spectra in Figures 1A and 3A is the smaller contribution of the antisymmetric COO^- stretching mode (at approximately 1575 cm^{-1}) to the latter spectra in D_2O. The polar domain contains 11 Glu and 8 Asp; the nonpolar domain contains only 1 and 3, respectively. In

the deconvolved curve of the D_2O sample (Figure 3B) it can be seen that the peak height intensity is somewhat greater at 1585 cm^{-1} (Asp) than at 1564 cm^{-1} (Glu). The opposite was seen in Figure 1B.

The deconvolved spectra were subjected to curve fitting, and the results are shown in Table I. The major band in D_2O is at 1655 cm^{-1} (43%), which has moved only slightly from its position in H_2O (1657 cm^{-1}, 54%), although it is now somewhat broader. This band in D_2O must be predominantly non-deuterium-exchanged α helix. If the band at 1655 cm^{-1} had contained much contribution from random structure, then there would have been a much greater difference seen between the two solvents. The amide I band due to random structure typically shows a decrease in frequency of approximately 5–10 cm^{-1} in going from H_2O to D_2O. The band at 1645 cm^{-1} (12%) in D_2O is due to random structure (exchanged), and the corresponding band in H_2O is buried under the 1657-cm^{-1} band, adding to its intensity. If this assignment were correct, then the 1657-cm^{-1} band should have an area of 43% plus 12%, 54% is observed. The band at 1636 cm^{-1} (19%) in D_2O can be assigned to β structure; in H_2O the band was found at 1641 cm^{-1} (14%), indicating rather complete exchange. Additional β structure comes from the band at 1677 cm^{-1} (10%), a high-frequency component characteristic of antiparallel β pleated sheets (Surewicz & Mantsch, 1988). The band at 1663 cm^{-1} in D_2O (12%) can be assigned to turns. This band is at a higher frequency in H_2O and is buried under the band at 1678 cm^{-1}. The overall conclusion for the structure of the nonpolar domain of cytochrome b_5 is α helix 43%, β structure 33%, turns 12%, and random structure 12%.

This nonpolar membrane-binding domain has been subjected to a prior structural analysis. Dailey and Strittmatter (1978) have examined the domain by circular dichroism in aqueous solution, organic solvents, detergents, and lipid vesicles. In aqueous solution the domain had 35% helical structure, and in lipid vesicles it had 45% helix and 25% β structure. The latter value is quite similar to our FT-IR determination. Dailey and Strittmatter (1978) also discussed their earlier Chou and Fasman calculations that predicted 24% α helix, 24% β turns, and 24% β sheet and suggested that a unique 10-residue-long, three consecutive β turn region could form a helical region, possibly 3_{10}. This would increase the "helical" content to that seen by circular dichroism.

Whole Cytochrome b_5 Molecule. The spectrum of the whole native cytochrome b_5 molecule is shown in Figure 4. There is a much more dramatic change in both the original and deconvolved spectra in going from H_2O to D_2O than was observed with the individual domains. Again, the deconvolved spectra were subjected to curve fitting, and the results are shown in Table I. Most of the bands present in the spectrum of the whole protein are seen in one or another of the two domains, as expected, and the assignments for the spectrum in D_2O are as follows: the 1683-, 1675-, and 1625-cm^{-1} bands are due to β structure (19%); the 1662-cm^{-1} band is due to turns (15%); and the 1651-cm^{-1} band is due to α helix (33%). The 1639-cm^{-1} band can contain random structure (exchanged), β structure, and perhaps the 3_{10} helix. To make an assignment for this band, the bands in the H_2O spectrum at 1642 and 1656 cm^{-1} need to be considered. As was pointed out in the discussion above concerning the polar and nonpolar domains, the 1642-cm^{-1} band (18%) can be due to 3_{10} helix, and β structure and the 1656-cm^{-1} band (46%) can contain contributions from α helix and random structure. The α-helical component upon H → D exchange should shift to approximately 1650 cm^{-1} in D_2O and a band at 1651 cm^{-1} is

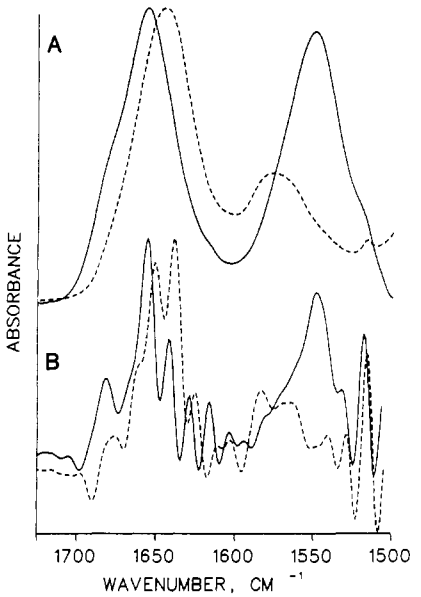

FIGURE 4: Infrared spectra of the whole cytochrome b_5 molecule in H_2O and D_2O in the region of the amide I and amide II bands (1700–1500 cm^{-1}). FT-IR spectra of cytochrome b_5 were obtained as described under Materials and Methods. In (A) are shown the original infrared spectra and in (B) the spectra after band enhancement by Fourier self-deconvolution. (—) Sample in H_2O buffer; (---) sample in D_2O buffer.

in fact seen in the spectrum of the whole cytochrome b_5 in D_2O. However, the 1651-cm^{-1} band only has a relative intensity of 33%, and it is likely that the "missing" 13% of the 1656-cm^{-1} band seen in H_2O was due to random structure, which upon exchange has shifted much further and has been included in the 1639-cm^{-1} band of the D_2O spectrum. The 1639-cm^{-1} band in D_2O must be a complex composite of several unresolved bands, and of the intensity seen in this band (33%) we suggest 13% is due to random structure (exchanged) and 20% is due to 3_{10} helix and β structure. We thus conclude that the overall structure of the whole cytochrome b_5 molecule is α helix 33%, β structure plus 3_{10} helix 39%, turns 15%, and random structure 13%.

The native cytochrome b_5 molecule has long been suggested to be composed of two domains that do not appreciably interact with each other (Visser et al., 1975; Tajima et al., 1978). This is supported by the data in Table I, where a composite spectrum, generated by addition of spectra from the two domains, was subjected to Fourier self-deconvolution and curve fitting. The result is quite similar to that seen with the whole intact protein, supporting the notion of the independence of the two domains. There are, however, some small, but probably genuine, differences between the actual spectrum and the composite spectrum in both D_2O and H_2O. From an examination of the spectra in D_2O the co-added sum of the isolated domains would seem to have slightly less intensity in the band at 1652 cm^{-1} than in the 1651-cm^{-1} band of the whole cytochrome b_5. From a comparison of the bands at 1656 and 1642 cm^{-1} in H_2O and the bands at 1651/1652 and 1639 cm^{-1} in D_2O, it would seem that in the composite spectrum the 10% difference between the 1656- and 1652-cm^{-1} bands appears in the 1639-cm^{-1} band and must be due to random structure, leaving 23%, the exact content of the 1642-cm^{-1} band in H_2O, to be β structure and 3_{10} helix. There is also an increase in the intensity of the high-frequency β bands (1683 and 1674 cm^{-1}) in the combined spectrum of the two domains compared to the spectrum of the native protein. From this comparison it appears that the two domains have slightly less α helix and

random structure and slightly more β structure (and/or 3_{10} helix) than is seen in the whole cytochrome b_5. It can also be noted that there is a slight, but probably genuine, difference in the position of the band assigned to α helix in D_2O solution: in the whole cytochrome b_5 it is at 1651 cm^{-1} and in the co-added domains at 1652 cm^{-1}. All these changes upon cleavage of the native cytochrome b_5 into its two domains are probably accountable by the aggregation state of the nonpolar domain versus that of the intact cytochrome b_5. It has been shown that the nonpolar domain alone forms a high molecular weight aggregate while the whole protein forms an octamer that is in equilibrium with monomeric protein (Calabro et al., 1976). It is possible that the differences in structure seen after the protein is cut into two domains are localized exclusively to the nonpolar domain because of this aggregation; certainly the aggregate would be less amenable to H → D exchange than a monomer–octamer system. But it is also possible that the close juxtaposition of the polar domains, forced together in the exterior of the octameric "supermicelle" (Calabro et al., 1976), causes a change in the structure of the polar domain. It should be noted that this octamer is an in vitro artifact; the concentration of the cytochrome b_5 in the cytoplasm does not get to these high concentrations used in FT-IR.

These small, yet detectable, differences seen in the infrared spectra point out the great sensitivity of FT-IR. It is hoped that an extension of this work will provide information on the structural changes that may occur upon membrane interaction of the protein, and it may also be possible to use accessibility to deuterium exchange to determine the amount of the nonpolar domain that is buried in the membrane after binding.

Registry No. Cytochrome b_5, 9035-39-6.

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