Infrared Spectroscopic Analysis of Salt Bridge Formation between Cytochrome b_5 and Cytochrome c†

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ABSTRACT: The infrared spectrum of a solution of a protein contains bands due to both the peptide backbone and the amino acid side chains. Generally, the bands due to the peptide backbone, between 1700 and 1600 cm^{-1}, are analyzed to determine the secondary structure of the protein; the bands due to the amino acid side chains, between 1600 and 1500 cm^{-1}, are largely ignored. When cytochrome b_5 is mixed with cytochrome c, under conditions that favor ionic complex formation, changes are seen in protein secondary structure and also in a band at 1562 cm^{-1}. The band at 1562 cm^{-1} is due to the side-chain carboxyl of Glu residues, rather than those of Asp residues that show a band at 1585 cm^{-1}, and the changes in the band at 1562 cm^{-1} indicate that when the two proteins interact, three ionized carboxyl groups of Glu become involved in salt bridge formation. This result is identical with that obtained by previous theoretical studies and suggests that infrared spectroscopy may be a rapid and quantitative method for the study of ionic interactions between proteins.

Cytochrome b_5 is found in the endoplasmic reticulum of animal cells and plays a central role in lipid metabolism. It is a component of the "microsomal electron transport chain" and functions as an intermediate electron carrier in several fatty acyl-CoA desaturases (Holloway, 1983), in fatty acyl-CoA elongation reactions (Nagai et al., 1983) and in sterol metabolism (Grinstead & Gaylor, 1982). Cytochrome b_5 also supplies electrons to cytochromes P-450, which are involved in other aspects of lipid metabolism and in xenobiotic metabolism (Tamburini & Schenkman, 1988). Besides these physiological electron acceptors, cytochrome b_5 will also react with the mitochondrial component cytochrome c. Cytochrome b_5 must have some general structural properties that enable it to interact with these various electron acceptors and, yet, achieve some specific orientation that enables electron transfer to occur.

In this paper, Fourier transform infrared spectroscopy (FT-IR) is used to monitor the ionic interaction of cytochrome b_5 with cytochrome c, and it is shown that this technique identifies and quantitates the side-chain residues that are involved in complex formation. This is the first application, to our knowledge, of FT-IR to the examination of protein–protein interactions by use of the spectral characteristics of the side chains of the amino acid residues. We suggest that this technique may be applicable to other systems in which ionic interactions between amino acid side chains, or other charged groups, have been postulated to occur.

An extensive series of studies have been performed by Strittmatter and co-workers (Dailey & Strittmatter, 1979, 1980) using chemical modification techniques to investigate the structural requirements for cytochrome b_5 interactions. These studies showed that the interaction of cytochrome b_5 with NADH–cytochrome b_5 reductase, stearoyl-CoA desaturase, or NADPH–cytochrome P-450 reductase involves the interaction between Glu residues 47, 48, and 52 and a single exposed heme propionate on the cytochrome b_5 and complementary Lys residues on the other proteins. Salemme (1976) proposed that complementary salt linkages are formed between negatively charged side chains on cytochrome b_5 and Lys residues on cytochrome c, and this suggestion has been supported by recent electrostatic analysis (Mauk et al., 1986) and by molecular dynamics simulations (Wendoloski et al., 1987).

FT-IR is being extensively used to investigate the structure of proteins and has recently been used to study cytochrome b_5 (Holloway and Mantsch, unpublished experiments). The major thrust of this study and others is the analysis of the conformationally sensitive amide I band. The amide I band of most proteins is a broad band located between approximately 1700 and 1600 cm^{-1} and is a complex composite of a number of overlapping bands characteristic for specific secondary structures, such as α helix, β structure, turns, and nonordered segments. These complex bands can be resolved to give an estimation of the secondary structure of the protein (Mantsch et al., 1986, Surewicz & Mantsch, 1988). Between approximately 1600 and 1500 cm^{-1} in the infrared spectrum there are several bands due to the amino acid side chains of the protein; however, when the sample is dissolved in H_2O, these bands are largely obscured by the strong amide II band around 1550 cm^{-1}. If the sample is prepared in D_2O, because of H → D exchange of the exchangeable N–H residues, the vibrations causing the amide II band are altered and, as a consequence of this, the amide II band is shifted to 1470 cm^{-1}. In D_2O, therefore, the bands due to side-chain vibrations are more evident. No great use has been made of these bands, although the studies of Chirgadze et al. (1975) on the free amino acids did enable some of the bands to be assigned to specific residues. We have recently extended these studies to peptides (Mantsch et al., unpublished experiments) and have clearly demonstrated that a band at 1564 cm^{-1} (antisymmetric COO^- stretching mode) is characteristic of peptides that contain Glu residues but is not seen with peptides that contain Asp residues; the latter show a COO^- stretching band at 1585 cm^{-1}. If the carboxyl group of the Glu residue in the peptide is titrated to the un-ionized form, the band shifts to 1710 cm^{-1}, as has been noted with poly(Glu) (Itoh et al., 1976), and a similar shift would also be expected if the carboxylate formed a salt bridge with a nearby Lys or Arg residue. This is the basis for our assay of the interactions between the two cytochromes.

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Materials and Methods

Cytochrome b_5 was prepared as described previously from rabbit liver (Markello et al., 1984). The protein was cleaved with TPCK–trypsin (in a ratio of 1 trypsin per 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) and gave a single band on gel electrophoresis. Before FT-IR analysis the protein was subjected to gel filtration on Sephadex G-25 in 10 mM NH_4HCO_3 and lyophilized. For convenience, this polar domain will be referred to as cytochrome b_5.

Cytochrome *c* (Sigma horse heart type III) was dissolved in D_2O and brought to pD 7.0 with HCl. Aliquots of this 1 mM solution were added to lyophilized samples of the polar domain of rabbit cytochrome b_5 to produce solutions that are 1 mM in cytochrome *c* and 0.67, 1.0, and 1.33 mM in cytochrome b_5. The solutions were left at room temperature overnight and were then assembled between CaF_2 windows separated by a 50-μm Teflon spacer. 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 coadded and Fourier transformed to give a resolution of 2 cm^{-1} with a signal-to-noise ratio of approximately 10000 to 1.

Results and Discussion

In order to observe the bands between 1600 and 1500 cm^{-1} due to side-chain vibrations, the amide II bands must be shifted from this region by exchange of the hydrogens of the N–H bonds of the peptide backbone. In our recent studies with cytochrome b_5 (Holloway and Mantsch, unpublished experiments), an analysis of the amide I and amide II bands in H_2O and D_2O indicated a complete H → D exchange of the peptide bonds had occurred within 1 h of exposure of the protein to the D_2O, with no change in the spectrum with longer times. In these present studies, a solution of cytochrome *c* in D_2O, in minimal salt, was added to lyophilized cytochrome b_5, and the samples were allowed to stand at room temperature overnight to ensure a stable level of H → D exchange had occurred.

Interferometric FT-IR measurements are made in a single beam mode; therefore, the infrared spectrum of each cytochrome *c*–cytochrome b_5 mixture was ratioed against a single beam spectrum of the original cytochrome *c* solution. This procedure produces a spectrum that is the spectrum of the mixture minus the spectrum of the original cytochrome *c* solution. The resultant infrared spectrum should be that of the cytochrome b_5 in the sample, provided no change has occurred in the spectrum of either component. These spectra are shown in Figure 1A, and although they have the same general profile as that of cytochrome b_5 itself (not shown), the ratios of the peak height absorbance of the band at 1575 cm^{-1} to that of the amide I band at approximately 1650 cm^{-1} (0.45, 0.41, and 0.38 for the solutions containing 0.67, 1.00, and 1.33 mM cytochrome b_5, respectively) are considerably less than the peak height ratio seen with cytochrome b_5 itself (0.54). This decrease must represent a loss of some functional group that generates a band near 1575 cm^{-1} in the original proteins. To quantitate this decrease, a spectrum of cytochrome b_5 was subtracted from each of the difference spectra shown in Figure

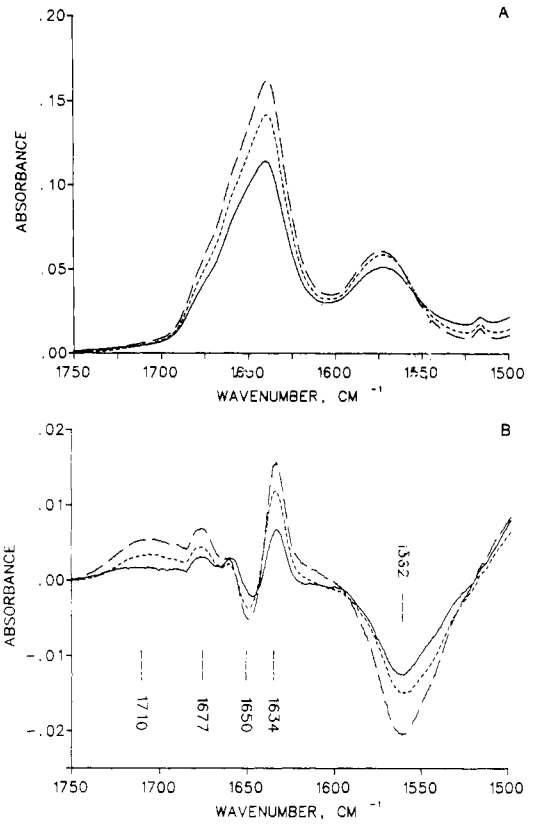

FIGURE 1: (A) Infrared spectra of cytochrome b_5 in the presence of cytochrome *c*. Cytochrome *c* (Sigma horse heart type III) was dissolved in D_2O and brought to pD 7.0 with HCl. Aliquots of this 1 mM solution were added to lyophilized samples of the polar domain of rabbit cytochrome b_5 to produce samples that were 1 mM in cytochrome *c* and 0.67 (—), 1.0 (---) and 1.33 (– –) mM in cytochrome b_5. FT-IR spectra were acquired as described under Materials and Methods, and from the spectrum of each cytochrome b_5–cytochrome *c* mixture the spectrum of the original cytochrome *c* solution was subtracted. (B) From each of the spectra shown in (A) was subtracted a "weighted" spectrum of cytochrome b_5, as described in the text, and the infrared difference spectra shown indicate the gains and losses in absorbance relative to what would have been seen in simple optical mixing of the two components.

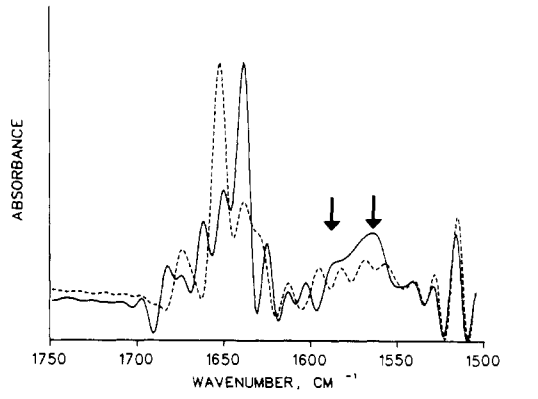

FIGURE 2: FT-IR spectra of cytochrome b_5 and cytochrome *c*. For comparison the spectra of the polar portion of cytochrome b_5 (—) and cytochrome *c* (---) (1 mM in D_2O) are shown. These were recorded as described under Materials and Methods and were subjected to Fourier self-deconvolution (Mantsch et al., 1986; Surewicz & Mantsch, 1988) by using a Lorentzian of 25-cm^{-1} half-bandwidth and a resolution enhancement factor (*k* value) of 2.5. The arrows indicate the positions of the antisymmetric COO^- stretching bands of Asp (at 1585 cm^{-1}) and of Glu (at 1562 cm^{-1}) in the cytochrome b_5 spectrum.

1A. Although the concentrations of cytochrome b_5 in each solution are known, it is not possible to subtract either the spectrum of a cytochrome b_5 solution of identical concentration or the spectrum of a solution attenuated by the calculated

amount, as the path length in the demountable cell varies slightly from sample to sample. Instead, the attenuation of the cytochrome b_5 spectrum, which was subtracted, was interactively varied until the absorbance of the resultant difference spectrum was the same at two positions where the absorbance was minimal in the original spectrum (1750 and 1520 cm^{-1}). These points were then assigned zero absorbance as shown in Figure 1B. In this figure a negative band indicates a loss of absorbance in the mixture and a positive band indicates an increment in absorbance in the mixture. The mean decrease in absorbance seen in the difference spectra at 1562 cm^{-1} for the three cytochrome b_5 concentrations is 0.017 ± 0.002 for a 1 mM solution in the 50-μm path-length cell. When the experiment was performed in 50 mM HEPES, there was no appreciable loss or gain in absorbance at any spectral position. Under these latter conditions the association constant for complex formation should be approximately 5000 times smaller than under low salt conditions (Mauk et al., 1986).

The spectra of the two cytochromes are shown, after band enhancement by Fourier self-deconvolution (Mantsch et al., 1986; Surewicz & Mantsch, 1988), in Figure 2. After deconvolution it can be seen that the band in the spectrum of cytochrome b_5 at approximately 1575 cm^{-1} is composed of at least two bands. In order to quantitate the decrease seen at 1562 cm^{-1} in Figure 1B, we decomposed the broad band seen at 1575 cm^{-1} in the spectrum of cytochrome b_5 by standard curve-fitting procedures into two bands at 1585 and 1562 cm^{-1} (indicated by arrows in Figure 2) and estimate the absorbance of the latter band to be 0.061 for a 1 mM solution of cytochrome b_5 in the 50-μm path-length cell. In the complex formation there has been a 28% decrease of the intensity of the band due to Glu residues, which suggests that 28% of the 11 Glu residues in cytochrome b_5, i.e., 3.1 residues, are involved in the formation of the complex. As mentioned earlier, three Glu have been implicated by prior studies.

It should be noted that this quantitation depends upon the number of the Glu that are in the ionized state, rather than in intramolecular cross bridges, in the original proteins. The above calculation assumes that all the Glu are ionized and contribute to the band at 1562 cm^{-1}. There is no quantitative evidence for this but three observations do support this contention. First, in a molecular graphics examination of cytochrome b_5 it appears that none of the Glu are sufficiently close to Lys or Arg to form intramolecular salt bridges. Second, in the original spectra of cytochrome c and cytochrome b_5 no band at 1710 cm^{-1} due to un-ionized carboxyl groups is seen. Third, in our unpublished spectra of the nonpolar and polar domains of cytochrome b_5, the ratios of the areas of the bands assigned to Tyr and Glu in these two fragments are in approximate agreement with their abundances in the two fragments, consistent with all the Glu being ionized in both fragments.

The infrared difference spectra in Figure 1B show additional features that, although we have not attempted to quantitate, are worthy of comment. The broad positive band at approximately 1710 cm^{-1} is in the expected position for an un-ionized COOD. In unpublished studies from this laboratory we have shown that the intensity of the band at 1564 cm^{-1} in the spectra of D_2O solutions (pD 7) of Glu-containing peptides decreases as the pD is lowered and a band appears at approximately 1710 cm^{-1}, due to the un-ionized COOD group (Itoh et al., 1976). This latter band is broad and has a much lower peak intensity than that of the original COO^-, as has been noted previously in studies with poly(Glu) (Chirgadze & Brazhnikov, 1974).

The positive band at 1634 cm^{-1} and the negative band at 1650 cm^{-1} are at positions usually assigned to β structure and α helix, respectively (Surewicz & Mantsch, 1988). The changes seen in these latter bands are suggestive of a loss of α helix with appearance of β structure. Although none of these changes can be assigned to one or the other protein, even the changes seen in Glu residues, it is known that cytochrome c is rich in α helix, and it is likely that the changes in the secondary structure are associated with the cytochrome c molecule, brought about by the strong complementary salt bridges. Coincidentally, the circular dichroism studies of Hlavica (1984) on the interaction of cytochrome b_5 with another α helix rich protein, cytochrome P-450, also showed a decrease in α-helical content when the two proteins were mixed together. The molecular dynamics calculations that have been performed on the cytochrome c–cytochrome b_5 system do indicate changes in protein structure upon interaction, in particular, the orientation of a Phe residue. However, this residue is in a region of extended polypeptide chain, and it seems unlikely that this motion would change the α-helical or β content of the system.

This first FT-IR study of the interaction between cytochrome b_5 and cytochrome c provides direct experimental evidence that three Glu's are involved in complex formation, as had been postulated from many other studies. The simplicity and quantitative nature of this technique suggest that it will be applicable to other systems where ionic interactions between proteins, or other systems, have been proposed to occur. This paper also emphasizes that FT-IR studies of proteins, or mixtures of proteins, can give valuable information besides the nature of the polypeptide backbone, and as there are much fewer side chains of a particular type than the total number of peptide bonds in a protein, such studies are inherently capable of greater quantitation.

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