Two-Dimensional Infrared Correlation Spectroscopy Study of the Aggregation of Cytochrome *c* in the Presence of Dimyristoylphosphatidylglycerol

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ABSTRACT Two-dimensional infrared correlation spectroscopy (2D-IR) was used in this study to investigate the aggregation of cytochrome *c* in the presence of dimyristoylphosphatidylglycerol. The influence of temperature on the aggregation has been evaluated by monitoring the intensity of a band at 1616 cm^{-1}, which is characteristic of aggregated proteins, and the 2D-IR analysis has been used to determine the various secondary structure components of cytochrome *c* involved before and during its aggregation. The 2D-IR correlation analysis clearly reveals for the first time that aggregation starts to occur between nearly native proteins, which then unfold, yielding to further aggregation of the protein. Later in the aggregation process, the formation of intermolecular bonds and unfolding of the α-helices appear to be simultaneous. These results lead us to propose a two-step aggregation process. Finally, the results obtained during the heating period clearly indicate that before the protein starts to aggregate, there is a loosening of the tertiary structure of cytochrome *c*, resulting in a decrease of the β-sheet content and an increase of the amount of β-turns. This study clearly demonstrates the potential of 2D-IR spectroscopy to investigate the aggregation of proteins and this technique could therefore be applied to other proteins such as those involved in fibrilogenesis.

INTRODUCTION

Fourier transform infrared spectroscopy (FTIR) is a well-suited technique to investigate the conformation of proteins because it provides information about their secondary structure. Either identification or quantification of each secondary structure component of a protein can be made from the analysis of the amide I band (Arrondo et al., 1993; Goormaghtigh et al., 1994; Dong et al., 1990; Sarver and Krueger, 1991; Surewicz et al., 1993) and, more rarely, from the amide II (Dousseau and Pézolet, 1990) and III (Anderle et al., 1987; Kaiden et al., 1987) bands in FTIR spectra. The amide I band, in particular, is very sensitive to changes in the protein secondary structure, and has been the object of several studies to evaluate the effect of lipid (Jackson et al., 1992; Sui et al., 1994), ligand binding (Baenziger et al., 1992), and temperature-induced unfolding or denaturation (Fabian et al., 1993; Williams et al., 1996; Surewicz et al., 1990).

Each conformation element, such as α-helices, β-sheets, turns, and random coils, has been associated to particular wavenumbers (Byler and Susi, 1986; Surewicz et al., 1993; Pribic et al., 1993; Goormaghtigh et al., 1994; Jackson and Mantsch, 1995) and can therefore be identified if the bands are well defined. However, most of the time these bands highly overlap, so that their identification is often difficult and sometimes impossible. Mathematical procedures, such as Fourier deconvolution (Kauppinen et al., 1981) and derivation (Cameron and Moffatt, 1984), have been developed to circumvent this problem.

More recently, two-dimensional infrared correlation spectroscopy (2D-IR) has been proposed by Noda (1989) to enhance the spectral resolution without assuming any lineshape models for the bands (Noda, 1990). Time-dependent variations in infrared spectra can be induced by an external perturbation, such as mechanical, thermal, chemical, electrical, or acoustic stimulations. A correlation analysis of these fluctuations generates two-dimensional maps that increase the spectral resolution by spreading peaks along the second dimension and that reveals the order of the actual sequence of processes induced by the perturbation (Noda, 1990). Although this technique has been mainly applied to polymers and liquid crystals, 2D-IR correlation analysis has also been used to identify the secondary structure components of proteins (Nabet and Pézolet, 1997; Pancoska et al., 1999; Kubelka et al., 1999), to investigate the mechanism of helix unfolding (Graff et al., 1997), and the heat-induced denaturation of proteins solubilized in water (Wang et al., 1998).

Cytochrome *c* is a peripheral protein involved in electron transport in the mitochondrial inner membrane although some studies have suggested that part of the protein could be slightly inserted in the membrane (Snel and Marsh, 1994; De Meulenaer et al., 1997), giving rise to hydrophobic interactions (Salamon and Tollin, 1996). Binding to negatively charged lipids is known to destabilize the cytochrome *c* structure since it decreases its denaturation temperature by 25 to 30°C and it increases its amide hydrogen-deuterium exchange rates (Heimburg and Marsh, 1993) and its structural unfolding process (Pinheiro et al., 1997; Sanghera and Pinheiro, 2000). The thermal denaturation of cytochrome *c*

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bound to negatively charged lipids such as dioleoylphosphatidylglycerol, dimyristoylphosphatidylglycerol (DMPG), and dipalmitoylphosphatidylglycerol, has been investigated by FTIR and has been found to induce the appearance of bands at 1616 and 1685 cm^{-1}, which have been attributed to hydrogen-bonded extended structures due to the aggregation of the unfolded membrane-bound proteins (Heimburg and Marsh, 1993; Muga et al., 1991a). The study of the ionic strength-dependence of the binding of cytochrome *c* to dioleoylphosphatidylglycerol dispersions has shown that the denatured proteins effectively tend to aggregate at the lipid surface (Heimburg and Marsh, 1995; Zhang and Rowe, 1994). It is also interesting to note that the interaction of apocytochrome *c* with negatively charged lipids stabilizes the α-helical content of the protein (Bryson et al., 2000) and induces the appearance of the bands characteristic of aggregated proteins on its FTIR spectra without any heating (Muga et al., 1991b). The aggregation of that heme-free precursor of cytochrome *c* is thought to facilitate its insertion in the membrane, which is necessary for its translocation across the outer mitochondrial membrane (Rietveld et al., 1986).

The study of protein aggregation is of primary importance since aggregation can be related to various phenomena, including amyloidosis and inclusion body formation (Speed et al., 1997). To eventually prevent or slow down protein aggregation, it is therefore of great interest to characterize the protein denaturation/aggregation process. In the present study, we have used Fourier transformed infrared spectroscopy to investigate the aggregation of cytochrome *c*, which has been considered as a good model of peripheral proteins for years, bound to negatively charged DMPG bilayers. More specifically, we have used the 2D-IR correlation technique to determine the cytochrome *c* secondary structure changes occurring before and during the aggregation process and most importantly, the sequence of the different events.

MATERIALS AND METHODS

Materials

Cytochrome *c* (type VI, oxidized form) was purchased from Sigma Chemical Co. (St. Louis, MO) and used without any further purification. DMPG was obtained from Avanti Polar Lipids (Alabaster, AL) and deuterium oxide (99.9%) was purchased from CDN Isotopes (Pointe-Claire, QC). Salts were of analytical grade.

Sample preparation

The samples for the infrared measurements were prepared by dissolving the amount of cytochrome *c* required to obtain the desired lipid:protein molar ratio (50:1) in 85 μl of buffer solution. This protein solution was then mixed with a Vortex mixer with 15 mg of dry DMPG. Lipid-protein complexes were then subjected to five freeze-thaw cycles to ensure homogeneity. The aqueous buffer solution used to prepare the samples was made of 50 mM HEPES, 40 mM NaCl and 1 mM EDTA, which was lyophilized, resuspended in deuterium oxide, and adjusted to p^2H 7.5. D_2O was used to study the amide I region free of any H_2O contribution (amide I′).

Infrared spectroscopy

Approximately 20 μl of each sample were injected between two previously heated BaF_2 windows, separated by a 13-μm Mylar spacer. All spectra (16 scans each) were recorded with a Nicolet Magna 550 spectrometer equipped with a narrow-band MCT detector. A homemade fast-purge system was used to allow early spectrum recording of injected samples. The Grams software (Galactic Industries Corp., Salem, NH) was used for the one-dimensional analysis of the 1720-1570 cm^{-1} spectral region. A linear baseline was subtracted from each spectrum and the amide I′ band has been normalized. For some 1D spectra, the amide I′ region, which consists of overlapping bands, was resolved by using Fourier self-deconvolution (Kauppinen et al., 1981; Cameron et al., 1982). This was done using the deconvolution software of Grams, which uses the deconvolution technique of Griffiths and Pariente, with a narrowing parameter (γ) of 7.82 and a smoothing parameter of 85% (Griffiths and Pariente, 1986).

The two-dimensional infrared correlation analysis was performed on normalized spectra, which were recorded before and during the cytochrome *c* aggregation. For these experiments, the spectrometer was thoroughly purged for at least 20 min in order to avoid correlation peaks due to the water vapor. For the aggregation study, the samples were kept at 40°C while the spectrometer was purged. The temperature was then rapidly increased up to the desired value and the spectra were recorded over different time periods.

To obtain the 2D-IR maps, heating was used as the perturbation to induce time-dependent spectral fluctuations in the IR spectra of DMPG: cytochrome *c* complexes due to the protein aggregation. The following procedure was used to calculate the 2D maps (Gericke et al., 1996; Nabet and Pézolet, 1997). The set of spectra can be expressed as a function of wavenumber ($\bar{\nu}$) and a running index (τ) in the time domain as $y(\bar{\nu}, \tau)$. The series of dynamic spectra $\tilde{y}(\bar{\nu}, \tau)$ were calculated by subtracting the first spectrum $y(\bar{\nu}, 0)$. The dynamic spectra can thus be expressed as:

$$\tilde{y}(\bar{\nu}, \tau) = y(\bar{\nu}, \tau) - y(\bar{\nu}, 0) \quad (1)$$

The N dynamic spectra were then discrete Fourier transformed, giving:

$$\tilde{Y}(\bar{\nu}, g) = \frac{1}{N} \cdot \sum_{\tau=0}^{N-1} \left(\tilde{y}(\bar{\nu}, \tau) \cdot \exp\left(-\frac{2i\pi\tau g}{N} \right) \right) \quad (2)$$

where g is the running index in the Fourier domain (Gericke et al., 1996). The synchronous ($\Phi(\bar{\nu}_1, \bar{\nu}_2)$) and asynchronous ($\Psi(\bar{\nu}_1, \bar{\nu}_2)$) correlations were obtained by using the following formula:

$$\Phi(\bar{\nu}_1, \bar{\nu}_2) = \frac{1}{\pi N} \cdot \sum_{g=0}^{N/2-1} (\mathrm{Re}\tilde{Y}(\bar{\nu}_1, g) \cdot \mathrm{Re}\tilde{Y}(\bar{\nu}_2, g) + \mathrm{Im}\tilde{Y}(\bar{\nu}_1, g) \cdot \mathrm{Im}\tilde{Y}(\bar{\nu}_2, g)) \quad (3)$$

$$\Psi(\bar{\nu}_1, \bar{\nu}_2) = \frac{1}{\pi N} \cdot \sum_{g=0}^{N/2-1} (\mathrm{Im}\tilde{Y}(\bar{\nu}_1, g) \cdot \mathrm{Re}\tilde{Y}(\bar{\nu}_2, g) - \mathrm{Im}\tilde{Y}(\bar{\nu}_2, g) \cdot \mathrm{Re}\tilde{Y}(\bar{\nu}_1, g)) \quad (4)$$

where Re and Im are the real and imaginary components of $\tilde{Y}(\bar{\nu}, g)$, respectively.

Correlation calculations were done in the amide I′ region (1695–1600 cm^{-1}) with the use of the Mathcad 7 Professional software for Windows (MathSoft Inc., Cambridge, MA). Visualization of the 2D-IR maps was

performed with the use of the Transform software (Research Systems, Boulder, CO).

RESULTS AND DISCUSSION

1D-IR spectroscopy

Spectra of cytochrome c in the absence and presence of DMPG

Fig. 1 *A* (solid line) shows the infrared spectrum in the amide I′ region of cytochrome *c* in solution at 30°C. In order to emphasize the main spectral features, the spectra were Fourier deconvolved (Fig. 1 *B*), a computational technique that decreases the width of the infrared bands (Kauppinen et al., 1981; Cameron et al., 1982). The Fourier deconvolved spectrum of cytochrome *c* in solution (Fig. 1 *B*, solid line) unravels three bands, a dominant band at 1653 cm^{-1} and two smaller bands at 1675 cm^{-1} and 1633 cm^{-1}. These results are in agreement with those obtained by Muga et al. (1991a) and Heimburg and Marsh (1993). The band at 1653 cm^{-1} is characteristic of the protein amide groups in α-helices (Surewicz and Mantsch, 1988) while the bands at 1675 cm^{-1} and 1633 cm^{-1} are generally associated with β-turns and β-sheets, respectively. However, cytochrome *c* is known to contain little β-structures (Provencher and Glockner, 1981; Takano and Dickerson, 1981; Dickerson et al., 1971) and overall, the protein is composed of three major and two minor α-helices (Bushnell et al., 1990). Therefore, it has been previously suggested that the bands at 1675 cm^{-1} and 1633 cm^{-1} can be attributed to either short, extended chains connecting the α-helices (Byler and Susi, 1986) or to helix-helix interaction (Reisdorf and Krimm, 1996).

Upon binding of cytochrome *c* to bilayers of DMPG, the overall contour of the cytochrome *c* amide I′ band is shifted by 2 to 3 cm^{-1} toward lower wavenumbers (Fig. 1 *A* and 1 *B*, dotted line). This is observed for both the gel and liquid crystalline phases of DMPG. These results are in agreement with those obtained by Muga et al. (1991a) and Hiramatsu and Yang (1983), who have interpreted the wavenumber shift to an overall loosening of the protein tertiary structure, which, in turn, results in an increased accessibility of the protein backbone to hydrogen-deuterium exchange. This perturbation of the tertiary structure is also reflected by a decrease in the thermostability of the protein, as discussed below.

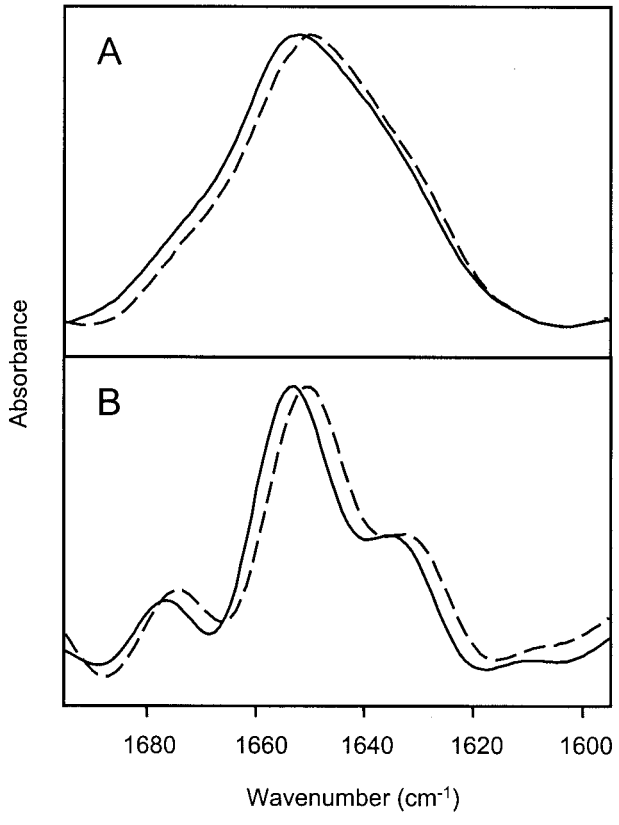

FIGURE 1 Original (*A*) and deconvolved (*B*) infrared spectra in the amide I′ region of cytochrome *c* in the absence (*solid line*) and the presence (*dotted line*) of DMPG (lipid:protein molar ratio, 50:1) at 30°C.

Effect of temperature on the DMPG-bound cytochrome c aggregation

Cytochrome *c* in solution has been shown to denature at a temperature of 83°C (Muga et al., 1991a). Protein denaturation is reflected in the infrared spectra by a broadening and a shift toward lower wavenumbers of the amide I′ band, and by the appearance of a well-defined band at 1616 cm^{-1}. This latter band is highly characteristic of thermally denatured proteins, and it has been previously assigned to hydrogen-bonded extended β-sheet structures between different protein molecules that are formed upon aggregation of thermally unfolded proteins (Surewicz et al., 1990; Jackson et al., 1991; Muga et al., 1991a). Furthermore, the denaturation temperature of cytochrome *c* has been shown to be at least 20 to 30°C lower in the presence of anionic phospholipids. In the case of the DMPG-cytochrome *c* complex at a lipid-to-protein molar ratio of 25:1, this denaturation temperature has been shown to decrease down to 56°C (Muga et al., 1991a; Heimburg and Marsh, 1993) while for the 50:1 complex further discussed below, the denaturation temperature of the DMPG-bound cytochrome *c* is 59°C (results not shown).

In the present study, we have investigated the aggregation and/or denaturation of DMPG-bound cytochrome *c* as a function of the time that the complex was kept at temperatures higher than its denaturation temperature. Fig. 2 shows an example of the evolution of the amide I′ band of cytochrome *c* in the DMPG-cytochrome *c* complex as a function of time at a temperature of 65°C. The results indicate an increase in the intensity of the 1616 cm^{-1} band and a decrease in the intensity of the 1650 cm^{-1} band as a

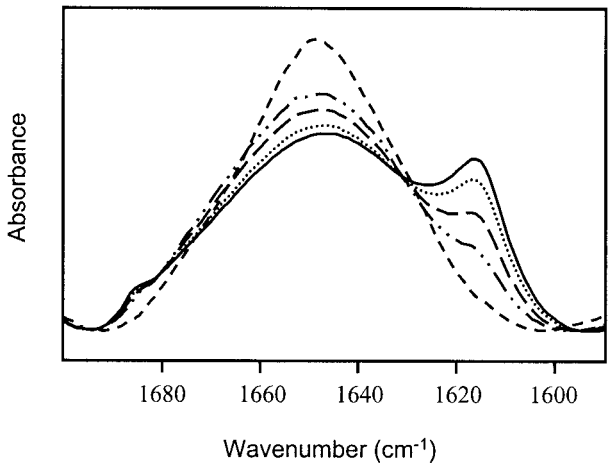

FIGURE 2 Infrared spectra of the amide I′ band of cytochrome *c* in the DMPG:cytochrome *c* complex (lipid:protein molar ratio, 50:1) kept at 65°C for 0 minute (*short dashed line*), 1 minute (*dashed and dotted line*), 3 minutes (*long dashed line*), 10 minutes (*dotted line*), and 120 minutes (*solid line*).

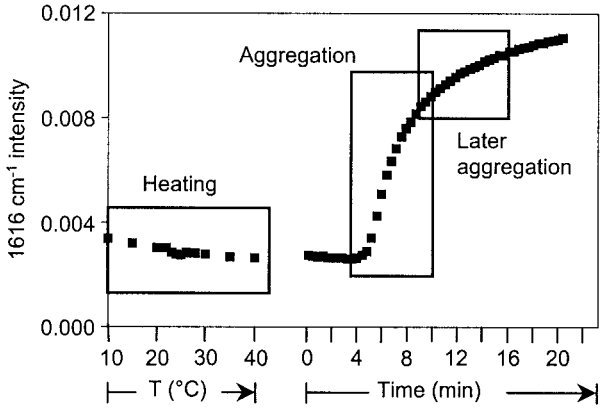

FIGURE 3 Schematic representation of the time periods used in the 2D-IR correlation analyses. The intensity of the 1616 cm^{-1} band was measured on spectra for which the amide I′ band has been normalized. The spectra were recorded as a function of increasing temperature before the aggregation and as a function of the time that the samples were kept at 65°C during the aggregation.

function of time, suggesting that aggregation of the proteins occurs. We have also investigated the effect of temperature on the rate of aggregation of DMPG-bound cytochrome *c*. The results (not shown) indicate that with increasing temperature, the final intensity of the 1616 cm^{-1} band is higher, which is indicative of a higher degree of cytochrome *c* aggregation. Finally, the reversibility of the aggregation process upon cooling and reheating of the complex has been monitored. No significant change in intensity was observed for the 1616 cm^{-1} band on the 1D-IR spectra during rapid cooling of the sample. However, the aggregation was found to be partly reversible with either the gel-to-liquid crystalline or the liquid crystalline-to-gel lipid phase transition, as long as the sample went through the transition slowly (results not shown). These results therefore suggest that the partial reversibility of the protein aggregation is modulated by the phase transition of the lipid. On the other hand, no reversibility was noted for the aggregated protein in the absence of DMPG (results not shown), as reported in the literature (Dong et al., 2000).

2D-IR

In order to get further insights into the secondary structure elements involved before and during the heat-induced aggregation of the DMPG-bound cytochrome *c*, 2D-IR analysis has been used. Fig. 3 shows the intensity of the 1616 cm^{-1} band as a function of increasing temperature (left) and as a function of time after the lipid-protein complex was rapidly brought at a temperature of 65°C (right). Synchronous and asynchronous maps were generated by 2D correlation analysis for each time period considered, as represented in Fig. 3. Rules used for the analysis of the sign of the peaks in these maps have been proposed by Noda (1990).

Aggregation

The synchronous map (Fig. 4, Φ) obtained for the DMPG-cytochrome *c* sample at the beginning of the aggregation period, as defined in Fig. 3, clearly shows that upon aggregation, important intensity changes occur at 1616 and 1650 cm^{-1}, wavenumbers that are characteristic of hydrogen-bonded extended structures and α-helices, respectively. The presence of a negative cross-peak between these two wavenumbers indicates that the changes occur in opposite directions, namely that the band at 1616 cm^{-1} increases whereas the one at 1650 cm^{-1} decreases upon aggregation. It can also be noted that the high-wavenumber component, characteristic of antiparallel extended structures, appears at 1685 cm^{-1}. On the other hand, the asynchronous map (Fig. 4, Ψ) shows one main cross-peak at 1616-1650 cm^{-1}. The negative sign of this cross-peak indicates that the formation of intermolecular bonds (band at 1616 cm^{-1}) starts to occur before the destabilization and/or unfolding of the α-helices. More specifically, the first intermolecular bonds created during the early aggregation are most likely formed between proteins which are nearly native or slightly destabilized due to their interaction with DMPG.

Later aggregation

We have also done the 2D correlation analysis on the later part, as defined in Fig. 3, of the DMPG-bound cytochrome *c* aggregation. The synchronous map obtained (Fig. 5, Φ) is very similar to that obtained for the beginning of the aggre-

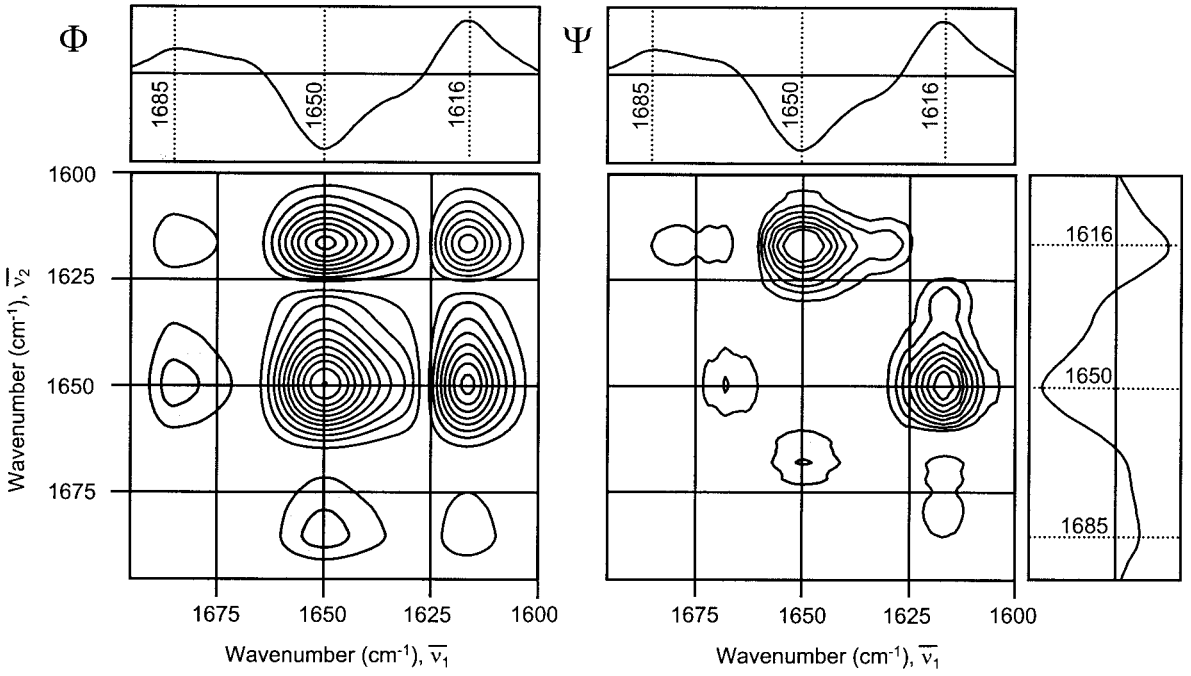

FIGURE 4 Synchronous (Φ) and asynchronous (Ψ) 2D-IR maps of DMPG-bound cytochrome *c* at the beginning of the aggregation. Clear and dark peaks are positive and negative, respectively. The one-dimensional spectrum shown in this figure is the average of the difference spectra used for the correlation analysis.

gation. Hence, the same increase in intensity is observed for the bands at 1616 and 1685 cm^{-1} while the band at 1650 cm^{-1} decreases. However, the asynchronous map (Fig. 5, Ψ) is strikingly different, and presents only a noisy pattern with no evidence of out-of-phase cross-peaks. These results can be interpreted as a simultaneous formation of intermo-

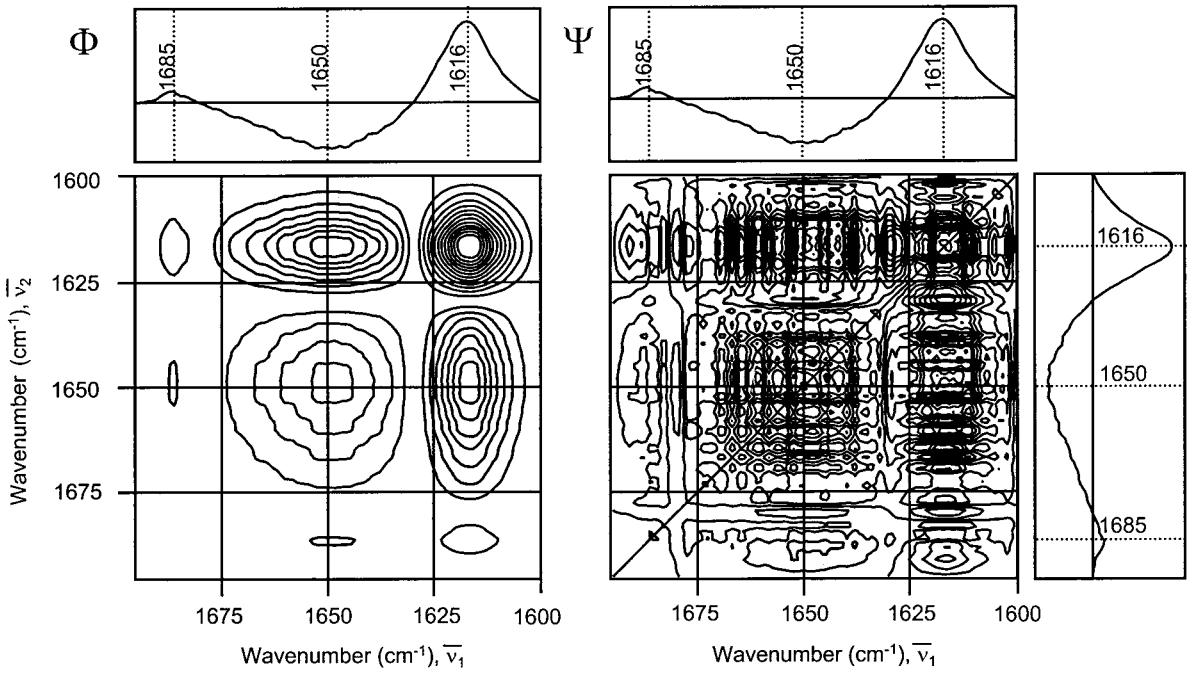

FIGURE 5 Synchronous (Φ) and asynchronous (Ψ) 2D-IR maps of DMPG-bound cytochrome *c* during the later part of the aggregation. Clear and dark peaks are positive and negative, respectively. The one-dimensional spectrum shown in this figure is the average of the difference spectra used for the correlation analysis.

lecular bonds and unfolding of α-helices in the later part of the aggregation.

A two-step aggregation/denaturation process

The results presented above suggest the presence of two different steps in the aggregation process of cytochrome *c*, which could in turn be related to the partial reversibility observed at the lipid phase transition temperature. As seen from the 2D-IR results, the early appearance of the 1616 cm^{-1} band is dependent on the protein aggregation and does not involve the unfolding of ordered secondary structures such as α-helices. This would be the fast and reversible aggregation process contributing to the band at 1616 cm^{-1}. On the other hand, the later part of the aggregation process is dependent on the kinetic of protein unfolding, thus denaturation (Surewicz et al., 1990; Jackson et al., 1991; Muga et al., 1991a). This would be the slower and irreversible aggregation process.

Heating

In order to understand how intermolecular bonds are first formed before the denaturation of any cytochrome *c* secondary structure components, we have investigated the evolution of the interaction between the protein and DMPG as a function of increasing temperatures but always below the denaturation point of the complex. It is already known that there is no important change in the cytochrome *c* secondary structure upon binding to DMPG (Muga et al., 1991a). However, we have made a correlation analysis on these spectra to find out if minor changes that could explain the increased amide group accessibility necessary for the formation of the first intermolecular bonds are occurring. The results indicate that slight changes in intensity occur upon increasing temperatures. As shown in the synchronous map (Fig. 6, Φ), the most important spectral changes occur at 1660 and 1625 cm^{-1}, and can be associated to β-turns and β-sheets, respectively. The negative cross-peak at these wavenumbers indicates that changes occur in opposite directions, namely that the intensity increases at 1660 cm^{-1} while it decreases at 1625 cm^{-1}, as it can also be noted from the one-dimensional average difference spectrum.

The asynchronous map (Fig. 6, Ψ) shows three main cross-peaks at 1653-1660 cm^{-1}, 1643-1653 cm^{-1} and 1625-1653 cm^{-1}. The signs of these peaks reveal that the first event to occur is the increase in intensity at 1653 cm^{-1}, followed by the apparently simultaneous increase in intensity at 1660 and 1643 cm^{-1} (extended chains) and the decrease in intensity at 1625 cm^{-1}. On one hand, the intensity increase at 1653 cm^{-1} may be explained by the appearance of a high-wavenumber α-helix component due to the loosening of the tertiary structure of DMPG-bound cytochrome *c* upon increasing temperature (Muga et al., 1991a). On the other hand, it seems that β-sheets are partially unfolded with increasing temperatures, causing an

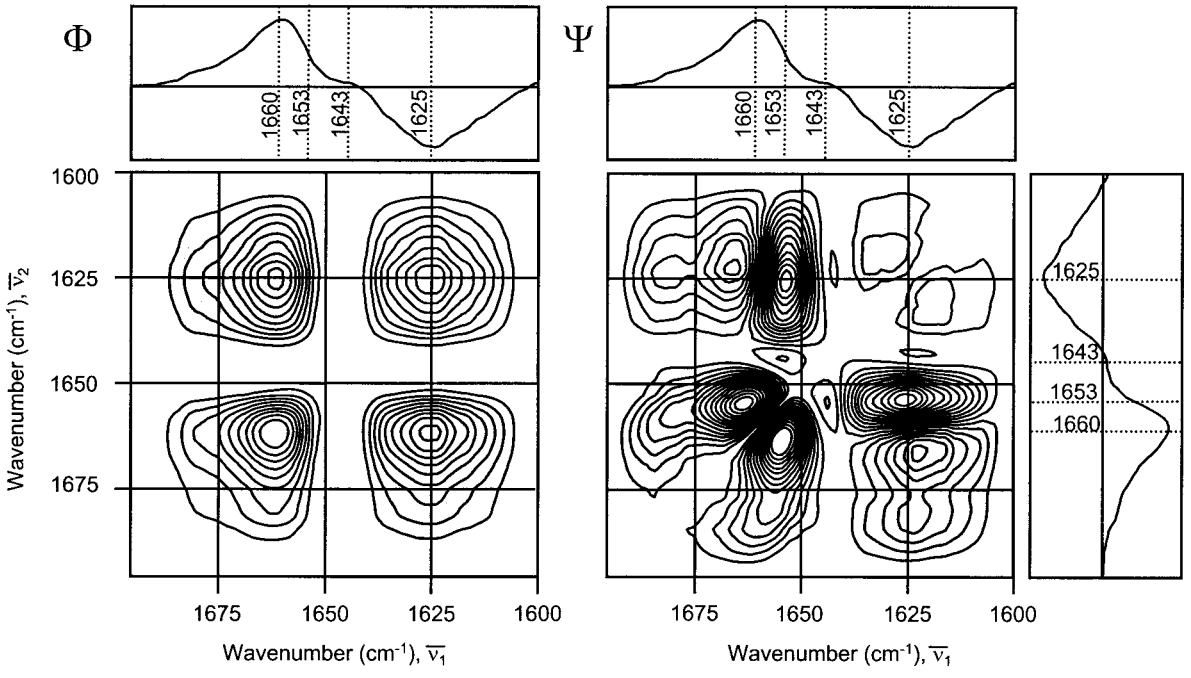

FIGURE 6 Synchronous (Φ) and asynchronous (Ψ) 2D-IR maps of DMPG-bound cytochrome *c* calculated from spectra recorded at temperatures increasing from 10 to 40°C (below the denaturation temperature of the complex). Clear and dark peaks are positive and negative, respectively. The one-dimensional spectrum shown in this figure is the average of the difference spectra used for the correlation analysis.

increase of β-turns and, to a lesser extent, extended chains. This can also be related to the loosening of the tertiary structure of cytochrome *c*, which leads to a greater hydrogen bond accessibility (Lo and Rahman, 1998). These results are interesting because they provide information about the most probable sites in which the formation of the first intermolecular bonds occurs at the beginning of the aggregation.

CONCLUSION

The results obtained in the present study indicate that 2D-IR correlation spectroscopy applied to the amide I band can provide novel and important information at the molecular level on the thermal denaturation mechanism of proteins. In particular, we have been able to identify that the beginning of the DMPG-bound cytochrome *c* aggregation process is facilitated by the loosening of its tertiary structure upon increasing temperature. Moreover, the secondary structure components involved in the denaturation/unfolding process of cytochrome *c* aggregation have been identified, as well as the chronological order of most of the events involved.

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