Conformational Changes in Proteins Induced by Low Temperatures: an Infrared Study

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Z. Naturforsch. **42c,** 1339–1342 (1987); received July 20/August 17, 1987

Hemoglobin, Myoglobin, Protein Conformation, FT-IR, Cryobiology

Infrared spectra of hemoglobin (met-hemoglobin) and myoglobin were recorded in the temperature range −110 °C to 30 °C. On cooling hydroalcoholic solutions of hemoglobin, the spectra indicate a conformational change (revealed by the appearance of a band at 1665 cm^{-1}) compatible with the appearance of distortions in its α-helical structure. In the case of myoglobin smaller effects are observed. These conformational changes are entirely reversible and do not occur in frozen aqueous solutions.

Biochemical and biophysical studies of proteins at low temperatures constitute a subject of considerable attention [1]. In particular, Douzou and coworkers have emphasized the need to work in fluid solutions at low temperatures [2]. However, there is practically no information on protein structure under these conditions [3]. We have initiated infrared spectroscopic studies aimed at the determination of the secondary structure of proteins at low temperatures. In this report we present results on the determination by infrared spectroscopy of conformational changes in fluid solutions of hemoglobin and myoglobin induced by cooling to −100 °C.

Materials and Methods

Human hemoglobin and sperm whale myoglobin were purchased from Sigma Chemical Co. (St. Louis, MO) and used as received. The proteins were dissolved in 20 mM phosphate buffer prepared in D_2O; for experiments in supercooled fluid media the buffer solutions were mixed with equal volumes of glycerol and the pD measured. Final protein concentrations were 1.5 mM. Infrared spectra were recorded at 2 cm^{-1} resolution on a Digilab FTS-15 Fourier transform spectrometer equipped with a HgCdTe detector operated at 77K. The protein solutions were held in cells of 50 μm pathlength fitted with BaF_2 windows. Spectra at various temperatures were recorded with the sample cell placed in a homemade evacuable chamber. The sample was cooled by circulating nitrogen gas from a liquid nitrogen container and the temperature controlled by the rate of cold nitrogen flow. The temperature was measured with a copper-constantan thermocouple in contact with the cell windows. Procedures for spectral analysis, deconvolution and derivation have been described elsewhere [4, 5]. Band narrowing by Fourier deconvolution was performed with Lorentzian lines of 15 cm^{-1} full width at half height and a resolution enhancement factor of 2 [4]. Fourier derivation was performed by using a power of 3 and a breakpoint of 0.3 [5].

Results and Discussion

Infrared spectra at various temperatures were recorded using both hydro-alcoholic mixtures (phosphate buffer/glycerol) and glycerol-free buffer solutions. In order to investigate the effects of cooling protein solutions we used mixtures of phosphate buffer with glycerol (1:1 vol/vol). The properties of hydro-alcoholic mixtures for low-temperature protein work have been described [3, 6, 7]. Spectra at low temperatures were recorded from solutions that had a pD 7 at room temperature. Since cooling increases the pD, control experiments were carried out with solutions at pD 8.2, the estimated pD at −100 °C [3] to separate possible pD-induced changes from cooling-induced changes. We also checked that the use of glycerol-buffer mixtures does not introduce spectral interference in the 1800–1600 cm^{-1} spectral region.

Fig. 1 shows the 1750–1600 cm^{-1} region of the infrared spectra of hemoglobin under different conditions. This spectral region contains the amide I bands which can be used to assess changes in the secondary structure of proteins [8]. The spectrum of hemoglobin dissolved in buffer-glycerol at 25 °C is seen in Fig. 1A and the spectrum of hemoglobin dissolved in glycerol-free buffer in Fig. 1C. Comparison of these two spectra allows the elucidation of possible effects induced by the presence of glycerol in the sample. In the spectrum recorded in buffer-

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0341–0382/87/1100–1339 $ 01.30/0

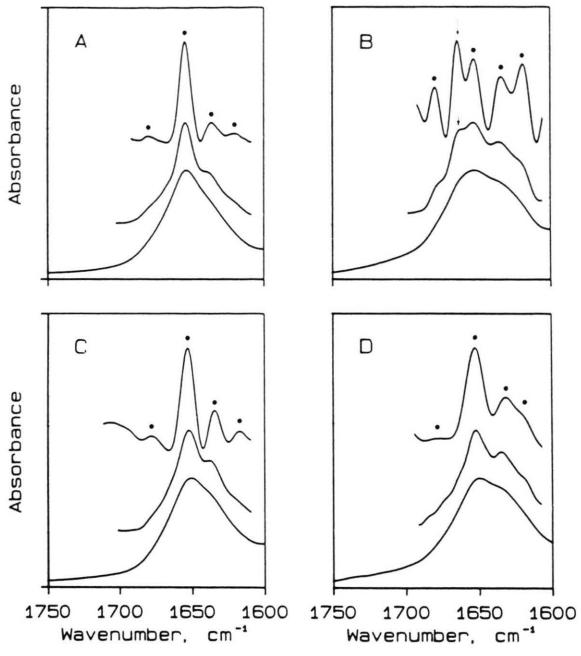

Fig. 1. Infrared spectra in the region of the amide I mode of 1.5 mM human hemoglobin: A. solvent: 1:1 glycerol-phosphate buffer (20 mM) prepared in D_2O, temperature: 25 °C; B. solvent: 50:50 glycerol-phosphate buffer (20 mM) prepared in D_2O, temperature: −100 °C; C. solvent: phosphate buffer (20 mM) prepared in D_2O, temperature: 25 °C; D. solvent: phosphate buffer (20 mM) prepared in D_2O; temperature: −100 °C. In all cases, the original spectra are shown as the bottom traces, the deconvolved spectra in the middle traces and the derivative spectra in the top traces. See Methods for details of the computational procedures.

glycerol (Fig. 1A) the deconvolved and derivative spectra indicate that the amide I band contour is comprised of a major band at 1655 cm^{-1} and three weaker bands at 1680, 1640 and 1621 cm^{-1} (labelled with ●). In the spectrum recorded in glycerol-free buffer (Fig. 1C) these bands are at 1680, 1653, 1638 and 1622 cm^{-1}. Detailed band assignments of infrared spectra of proteins are not the subject of this report and may be found in several publications [8–10]. It suffices to say that these bands are compatible with a structure of hemoglobin consisting primarily of α-helices which is in accord with previous investigations [9]. The presence of glycerol has only a negligible effect on hemoglobin. The two intense bands at 1655 and 1640 cm^{-1} are shifted 2 cm^{-1} to lower wavenumbers when the solvent does not contain glycerol. These differences are most likely due to the different polarity of the two media as the hydro-alcoholic solutions are less polar than the aqueous buffer solutions. Yet it is clear that the presence of glycerol does not bring about drastic changes in the secondary structure of the protein and therefore studies in hydro-alcoholic media are relevant to our understanding of changes produced under physiological conditions. This is in accord with the findings of Douzou and coworkers [11].

Fig. 1B and 1D show the infrared spectra at −100 °C of hemoglobin in glycerol-buffer and glycerol-free buffer, respectively. The spectrum recorded in glycerol buffer (Fig. 1B) differs considerably from that recorded in the same solvent at 25 °C (Fig. 1A). The overall band contour is much broader at low temperature and the deconvolved and derivative spectra demonstrate the appearance of at least one new band (labelled with an ↓) as well as small shifts of the other bands; bands are now observed at 1680, 1665, 1654, 1634 and 1620 cm^{-1}. Examination of infrared difference spectra (not shown) reveals that the new band at 1665 cm^{-1} appears at the expense of the strong band at 1654 cm^{-1}. These changes are brought about by the temperature effect and not by the associated increase in pD induced by cooling; the spectrum of hemoglobin at pD 8.2 (recorded either in glycerol or glycerol-free buffer) is essentially the same as the spectrum recorded at pD 7. The spectrum shown in Fig. 1B was recorded after the sample had been at −100 °C for 45 minutes and we also note that no further changes were observed over a period of two hours at −100 °C.

One important aspect of the present results is that the spectral changes observed on cooling are entirely reversible, *i.e.*, the spectrum obtained after the sample is allowed to warm to 25 °C is identical to that prior to cooling. Also, the changes observed do not seem to occur suddenly at a critical temperature. Analysis of the spectra recorded at intermediate temperatures between 25 °C and −100 °C reveal a gradual appearance of the band at 1665 cm^{-1}. However, on warming, the spectra indicate a rather abrupt disappearance of the 1665 cm^{-1} band between −60 and −50 °C.

Fig. 1D shows the spectrum of hemoglobin dissolved in glycerol-free buffer at −100 °C. From the deconvolved and derivative spectra in Fig. 1D a major band is found at 1652 cm^{-1} and three weaker bands at 1675, 1635 and 1618 cm^{-1}. While there are

small shifts induced by cooling with respect to the spectrum in Fig. 1C, no new bands appear on cooling. This is in marked contrast to the case of the hydro-alcoholic solution (compare Fig. 1A and 1B).

From previous studies it is known that, in infrared spectra of proteins in D_2O solutions bands in the 1668 to 1662 cm^{-1} region correspond to turn-type structures [12–14]. Therefore, it could be suggested that the appearance of a band at 1665 cm^{-1} in the hemoglobin spectra on cooling in a fluid medium indicates the formation of turns at the expense of some α-helical structures. It should be stressed, however, that the appearance of the band at 1665 cm^{-1} is entirely reversible and it seems doubtful that such a process should be reversible. Furthermore, such a process involves unfolding of the α-helices to be refolded as turns. This implies that in D_2O solution the residues involved would undergo H/D exchange and therefore the spectrum obtained after re-warming should be different, *i.e.*, characteristic of an α-helix with deuterium exchanged NH groups. Therefore, we propose that the changes observed represent a structural modification of the α-helices. The wavenumber observed (1665 cm^{-1}) is compatible with a α-helix with a different hydrogen bonding pattern and it could be proposed that a 3_{10} helix is formed. Interestingly, recent calculations give a wavenumber of 1665 cm^{-1} for the 3_{10} helix in poly-(α-aminoisobutyric)acid [15]. Infrared bands at 1662 cm^{-1} have been found in the spectra of bacteriorhodopsin and attributed to distorted α-helix rods [16]. The fact that the structural change discussed above is only observed at low temperature in fluid solution and not in frozen solution probably indicates that such a change is only feasible in a medium allowing the protein a certain degree of flexibility. At the same time, the effects observed could be due to the presence of glycerol in the medium; if this were the case glycerol would have to interact only at low temperature since at room temperature the spectra recorded in glycerol-buffer and glycerol-free buffer are practically identical (*cf.* Fig. 1A and 1C).

The effects observed on cooling hemoglobin in the presence of glycerol could also be due to the fact that this protein has quaternary structure which may be changing as the temperature decreases. In order to test this possibility we have recorded spectra of myoglobin, with no quaternary structure and whose secondary structure is similar to that of hemoglobin [17].

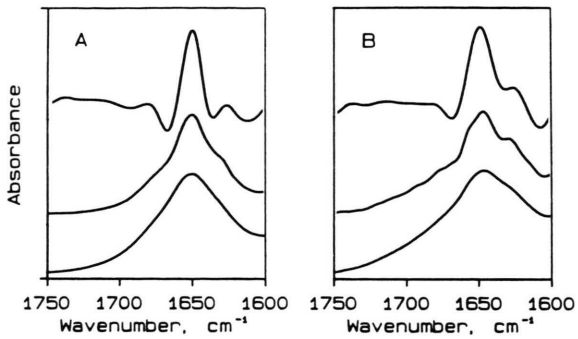

Fig. 2. Infrared spectra in the region of the amide I mode of 1.5 mM sperm whale myoglobin dissolved in 50:50 glycerol-phosphate buffer (20 mM) at 25 °C (A) and at −100 °C (B). See caption to Fig. 1 for details of the presentation of spectra.

Fig. 2 shows the amide I region (1750 to 1600 cm^{-1}) of the infrared spectrum of myoglobin dissolved in buffer-glycerol at 25 °C (Fig. 2A) and at −100 °C (Fig. 2B). From these spectra it is seen that the effect of cooling the myoglobin solution to −100 °C is not the same as that of cooling the hemoglobin solution (Fig. 1B). In the case of myoglobin, the main band due to the α-helices appears at 1652 cm^{-1} at 25 °C. At −100 °C there appear two bands at 1657 and 1648 cm^{-1} and the "new" strong band at 1665 cm^{-1} observed in the spectrum of hemoglobin at −100 °C (Fig. 1B) is absent in the spectrum of myoglobin at −100 °C (Fig. 2B).

The behaviour of myoglobin is thus different from that of hemoglobin; since these two proteins differ mainly in the fact that hemoglobin has quaternary structure it is possible to speculate that the conformational change observed in the case of hemoglobin is a consequence of cooling-induced alterations to its quaternary structure. In the case of myoglobin, with no quaternary structure, the spectral effects observed probably reflect slight modifications to its α-helices.

Acknowledgements

U. K. thanks the Alexander von Humboldt Stiftung for a Lynen fellowship. This work was supported in part by CAICYT (Spain) and by a NATO Travel Grant. Issued as NRCC publication No. 27494.

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