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The Use of Deuterium Exchange for the Study of the Distortion of α-Helices in Proteins

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The geometrical distortion of the α-helical structure of the globular proteins sperm-whale myoglobin, bacteriophage T4 lysozyme and hen egg-white lysozyme have been studied by means of deuterium exchange in solution. It was examined with the use of infrared spectroscopy in the region of the amide A band. The parameters of this band are known to be dependent on the length and geometry of the peptide hydrogen bond. In this way an estimation of the structural heterogeneity of the polypeptide backbone of the protein molecule has been achieved by studying the half-width of the amide A band during successive deuteration of the protein in heavy water solution. For all the proteins studied the peptide groups with broad amide A bands were exchanged at the first stage. These groups have been assigned as belonging to the unordered form of the molecule. The α-helical fragments were assigned to have smaller values of half-widths of the amide A band, and these were exchanged at the second stage. From these data α-helical fragments were shown to be characterized by a set of geometrical distortions. The results obtained also disclose a correlation between the degree of geometrical distortion of α-helical structure in the protein molecule and the dynamic accessibility of their peptide groups to a water molecule.

1. Introduction

At present, practically all available evidence on the details of the spatial structure of proteins at high resolution has been obtained by means of X-ray structural analysis. In these cases the accuracy of determination of the atomic positions for the main chain is about a few tenths of an ångström unit (Moews & Kretsinger, 1975), and the length of the peptide hydrogen bond has been determined with an accuracy of about 0·3 Å. A corresponding error in the angles of internal rotation of the polypeptide chain is equivalent to about 15°. These errors are too large for the accurate estimation of small distortions in the structure of the polypeptide chain. From the stereochemistry of the polypeptide chain and also from a consideration of the known spatial structure of proteins it can be concluded that the polypeptide backbone of regular fragments of protein molecules should be distorted; however, direct experimental evidence for this is lacking. In a previous paper we showed that infrared spectroscopy in the region of amide vibrations allows us to observe the geometrical distortions of helical structures (Chirgadze *et al.*, 1976). In this paper the distortions of the geometry of peptide hydrogen bonds in the α-helical fragments of some globular proteins are studied. The analysis of distortions is based on the study of the amide A

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band half-width for the protein solutions in heavy water, using the procedure of successive deuteration described for the first time by Chirgadze (1972).

2. Materials and Methods

(a) *Materials*

A chromatographically pure preparation of sperm-whale myoglobin in met-form was prepared according to Hapner *et al.* (1968). The protein/ammonium sulphate paste was dissolved in 0·05 M-phosphate (pH 5·6) and dialysed against the same buffer. Bacteriophage T4 lysozyme was isolated by A. V. Troitsky according to a modification of the method of Tsugita *et al.* (Tsugita *et al.*, 1968; Troitsky & Lysenko, 1975). The purified enzyme migrated as a single band during polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate, and 2 bands running close together (active forms I and II) in the β-alanine system at pH 4·5. The hen egg-white lysozyme was obtained from Serva, and was recrystallized 3 times. The deuterated reagents were from Isotop, U.S.S.R., and had a basic isotope purity of 99·8% for D_2O and 99% for DCl.

(b) *Methods*

(i) *Preparation of solutions*

The protein solutions were prepared by dissolving freeze-dried preparations in heavy water with appropriate pD values and ionic strength. In the acid region, the pD of water was brought to 0·1 N-DCl. The procedure of dissolving and filling the cell took 2 to 3 min. The type of cell used and the technique for measuring infrared spectra in heavy water solutions were described earlier (Chirgadze *et al.*, 1973). The work in the region of the amide A band requires a cell with a thickness of about 120 μm and a protein concentration of 1 to 2%. The measurements were taken in the temperature interval 5°C to 65°C using thermostatically controlled cells. The concentration of solutions was determined with a Hitachi EPS-3T spectrophotometer, using the known extinction coefficients. At neutral pH the following values were taken: sperm-whale myoglobin, $E^{1\%}_{1\,cm} = 89{\cdot}6$ at 409 nm; bacteriophage T4, $E^{1\%}_{1\,cm} = 12{\cdot}8$ at 280 nm; and hen egg-white lysozyme, $E^{1\%}_{1\,cm} = 26{\cdot}9$ at 280 nm. pD values of solutions were measured with a pH meter and then corrected by +0·4.

(ii) *Measurement of infrared spectra*

The spectra were recorded with a Perkin-Elmer 180 spectrophotometer. The spectral width of the slit at 3300 cm^{-1} was 6 cm^{-1}. The expanded optical density scale was used so that the complete scale corresponded to 0·3 optical density units. Linearity of the device was checked by rotating calibrated discs, and it was not worse than 1%. The half-width of the amide A band was determined with an accuracy of about 2 to 3 cm^{-1}. The errors in the determination of the half-width of the difference bands varied from 4 to 12 cm^{-1}, depending on the value of the half-width. The isotopic composition in the reference cell was adjusted before measurements were made such that the quantity of light water was equal to or a little more than the quantity of light water in the sample cell. The isotopic composition was checked by the band of OH-stretching vibrations near 3400 cm^{-1}. The recording of the spectrum in the region of 3700 to 2800 cm^{-1} took about 10 min. Successive deuteration of proteins was carried out by heating the solution for a short time at temperatures not exceeding the temperature of denaturation.

(iii) *The treatment of spectra*

In the process of protein deuteration the hydrogen atoms of peptide groups are exchanged for deuterium atoms from heavy water. This leads to the deterioration of isotopic composition in the sample cell, as a result of which the band intensity of OH-stretching vibrations of the water molecule in the form HOD near 3400 cm^{-1} increases, and the band partially overlaps with the protein amide A band. We obtain pure amide absorption in the region of 3000 to 3500 cm^{-1} by subtracting the band of water in such a way that its

contribution to the spectrum in the region of 3500 to 3700 cm^{-1} is zero. The parameters of the contour of this band were obtained at arbitrary discompensation in the region of the OH band near 3400 cm^{-1} for 2 similar cells with heavy water. The integral intensity of the amide A band was determined according to the formula (Chirgadze *et al.*, 1973)

$$B = 2{\cdot}3\, K(f_g)\, \varepsilon_0\, \Delta\nu_{1/2},$$

where f_g is the parameter of the contour shape, ε_0 is the band intensity at maximum, and $\Delta\nu_{1/2}$ is the band half-width. In all cases the parameter of the contour shape was close to a value of 0·5, and the corresponding constant is $K(f_g = 0{\cdot}5) = 1{\cdot}32$.

3. Results

(a) *Estimation of the distribution of geometrical parameters of peptide hydrogen bonds by the half-width of the infrared amide A band*

The amide A band is assigned to the stretching vibration of the NH-bond of the peptide group at which practically only the hydrogen atom is considerably displaced. The NH-bond vibration displays two bands in the infrared spectrum: the amide A band near 3300 cm^{-1} and the amide B band near 3100 cm^{-1}. The splitting results from resonance interaction of a Fermi type of the fundamental tone of NH-vibration with a frequency near 3300 cm^{-1}, and of the first overtone of the amide II band, the frequency of which is approximately equal to $1550 \times 2 = 3100$ cm^{-1} (Miyazawa, 1960). The intensity of the fundamental amide A band includes 80 to 90% of the overall intensity of NH-stretching vibration. Therefore we can consider, as a good approximation, that the properties of this group are wholly determined by the amide A band.

The values of frequencies and half-widths of the amide A band for different conformations of the main chain of the model polypeptides and some fibrous proteins have been given elsewhere (Kobyakov, 1969; Chirgadze *et al.*, 1976). The amide A band frequency for α-helical structures is close to a value of 3300 cm^{-1} and its half-width can be in the range of 56 to 90 cm^{-1}. In the case of the pleated sheet, the value of this band frequency is in the range of 3266 to 3300 cm^{-1}, and its half-width can vary within the same limits. In the case of the unordered form the band frequency can vary in the range of 3250 to 3305 cm^{-1}, and the half-width is 150 to 200 cm^{-1}.

The intensity of this band can serve as a rather good indicator of the number of peptide groups. For example the amide A band intensity of the helical polypeptide poly(γ-benzylglutamate) in chloroform is equal to $4{\cdot}2 \times 10^4$ l mol^{-1} cm^{-2} (Chirgadze & Rashevskaya, 1969). We estimated the amide A band intensity of the antiparallel pleated sheet by the spectrum of poly(*S*-carbobenzoxymethyl cysteine) solution in dichloroethane with a small addition of trifluoroacetic acid, when the polypeptide is in 80% ordered form: it was equal to $4{\cdot}8 \times 10^4$ l mol^{-1} cm^{-2}. We failed to estimate the amide A band parameters in unordered form in solution. An approximate estimation of this band intensity was obtained from the spectrum of a film of silk fibroin from *Bombyx mori*, it appeared to be $4{\cdot}5 \times 10^4$ l mol^{-1} cm^{-2}. On the basis of these data we will consider that the amide A band intensity has a value of $4{\cdot}5 \times 10^4$ l mol^{-1} cm^{-2} independent of peptide chain conformation.

Spectral parameters of the absorption band of NH-vibrations are closely connected with many physico-chemical and geometrical properties of the hydrogen bond NH . . . O (Pimentel & McClellan, 1960). For hydrogen bonds of different types XH . . . Y empiric dependences have been established between the frequency of XH-stretching vibrations and the hydrogen bond length, i.e. the distance X . . . Y

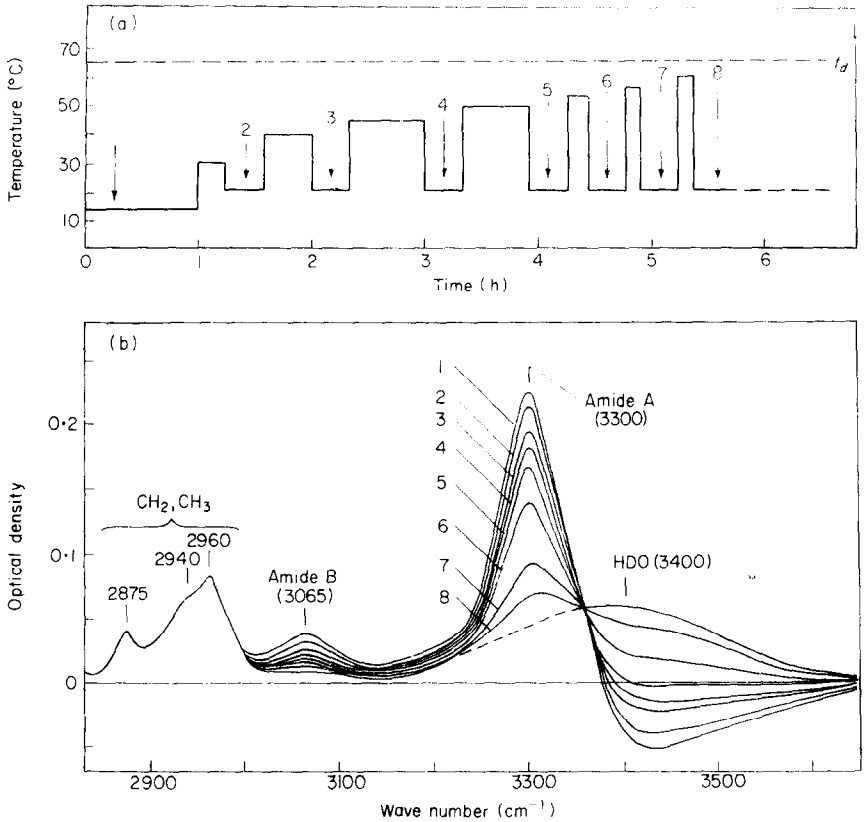

FIG. 1. An example of experiment involving successive deuteroexchange of bacteriophage T4 lysozyme solution in heavy water, 0·001 M-KCl (pD 4·4).

(a) Scheme of the experiment. t_d, denaturation temperature.

(b) The experimental curves of the infrared spectrum in the region of the amide A band after the corresponding procedure of successive deuteroexchange. The numbered arrows correspond to the different steps of deuteroexchange in (a) and (b).

(Pimentel & Sederholm, 1956). Therefore, in the case of a set of hydrogen bonds with different lengths and distortions, the NH-stretching vibration band must be broadened. We know that this effect is observed in the spectra of polypeptides and fibrous proteins (Chirgadze *et al.*, 1976). Thus, the amide A band half-width or, to be more exact, the additional broadening in the range of half-widths larger than 60 cm^{-1}, can serve as a criterion of geometrical distortions of peptide hydrogen bonds NH . . . O.

Let us consider a method of estimation of hydrogen bond distortions of the main chain in the proteins which is based on this phenomenon. The principle of the method consists of studying the dependence of the amide A band half-width of protein dissolved in heavy water on the degree of deuteroexchange. The idea of such an approach was first employed for structural interpretation of separate parts of a myoglobin molecule on the basis of accessibility of heavy water molecules (Chirgadze, 1972), using wet films of protein. In this paper we have applied this method to the study of structural heterogeneity of proteins in solution. The band half-width for the groups being exchanged at any given moment can be determined from the difference spectra

of successive exchange. The number of peptide groups subjected to exchange is determined by the amide A band intensity. The dependence of the amide A band half-width of the difference contours on the total amount of exchanged groups allows an estimate to be made of the distribution of groups with the given half-widths in a molecule.

As an example of the experimental procedure, Figure 1 presents a plot from an experiment involving successive deuteroexchange in phage T4 lysozyme and shows the corresponding infrared spectra. During this experimental procedure, as well as the amide A band an additional band of isotopic water appears. This band has a frequency maximum near 3400 cm^{-1} and a half-width of about 270 cm^{-1}. It almost overlaps the amide A band. An exact measure of isotopic water was made by evaluating its quantity from the absorption at 3500 cm^{-1}, followed by subtraction of the corresponding absorption contour. The latter was determined for the completely deuterated protein. This contour was naturally the same for solutions of different proteins.

The rate of deuteroexchange in globular proteins depends essentially on temperature and pD (Englander *et al.*, 1972). This value is at a minimum at a temperature of solution near 0°C and in the pD range 2 to 4. The technique of temperature-jump was used to carry out successive deuteroexchange. The temperature was increased for a certain period of time by 20 to 30 deg. C without, however, reaching the region where thermal denaturation starts. The measurements were made at 15 to 20°C. The procedure of exchange was done in such a way that at every successive measurement the number of exchanged peptide groups increased by 5 to 10%. In the majority of cases the method described permitted the deuteration of 85 to 95% of the groups of a protein molecule. On the other hand, to study the rapidly exchanged part of the molecule it is necessary to work in the region of pD 2 to 4 and at a lower temperature, 5 to 10°C. The root-mean-square error in determining the number of groups was 3 to 5% of the total groups in the molecule.

The contours of the amide A band, corrected for absorption of isotopic water, and the corresponding difference curves are presented in Figure 2. Thus, we can plot the dependence of the amide A band half-width of successively exchanged NH peptide groups on the total amount of exchanged groups in the protein molecule.

(b) *Estimation of distortions of peptide hydrogen bonds in some proteins*

The obtained dependences of the amide A band half-width on the degree of deuteroexchange for a number of proteins with a known spatial structure are given in Figure 3. The spectra were measured for solutions of sperm-whale myoglobin, phage T4 lysozyme and hen egg-white lysozyme in heavy water. Sperm-whale myoglobin at room temperature starts to denature at a pD value of about 5·0. For this protein all measurements were carried out at pD 5·6 and at a temperature of 20°C, except for the first two which were performed at 5°C. Under these conditions the exchange of the first 20 to 30% of peptide hydrogens is complete in only a few minutes, therefore in our experiments we could not record the degree of exchange of less than 25%. For the same reason, the curves for the other proteins are not given from the beginning.

The structures of these proteins were obtained earlier at a resolution of 1·4, 2·4 and 2·0 Å (Watson, 1969; Matthews & Remington, 1974; Imoto *et al.*, 1972). The

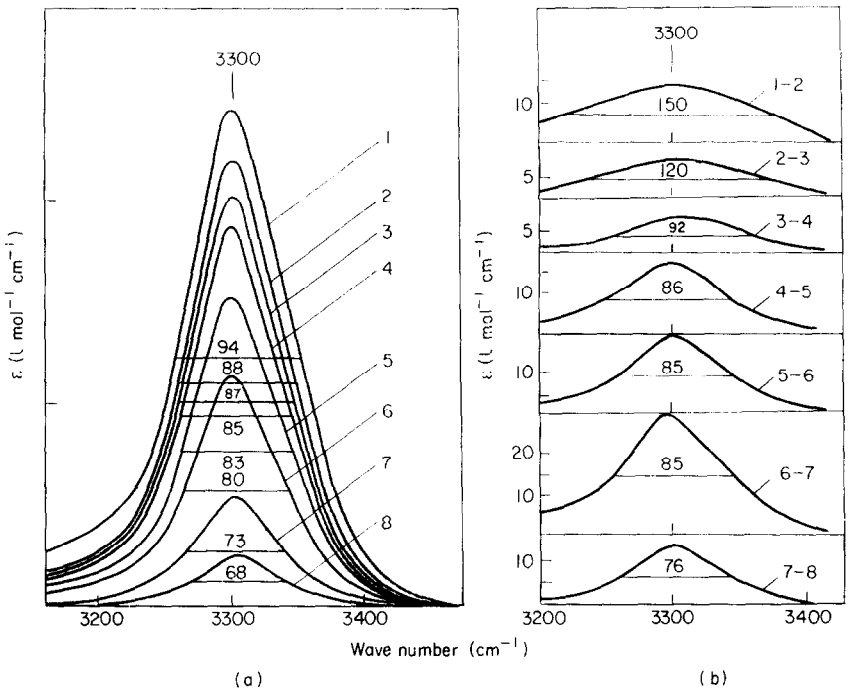

FIG. 2. Infrared spectra of bacteriophage T4 lysozyme solution in heavy water, 0·001 M-KCl (pD 4·4).

(a) The contour of the amide A band at different degrees of deuteroexchange: the numbers refer to Fig. 1.

(b) Difference contours between the neighbouring curves.

α-helix content in sperm-whale myoglobin calculated from X-ray data by the number of peptide groups is 74%. The α-helix content in phage T4 lysozyme amounts to 52%; there is also one small fragment of pleated sheet with a total amount of peptide groups of about 8%. In hen egg-white lysozyme the helical fragments contain about 40% of the peptide groups; there is one fragment of pleated sheet, its relative content is equal to 9%.

It is characteristic of all the proteins studied that the groups with a large half-width of 100 to 200 cm^{-1} are easily exchanged, while the groups exchanged with difficulty have a half-width of 70 to 90 cm^{-1}. When analysing these curves we can use the fact that the half-width of ordered fragments covers the interval of 60 to 90 cm^{-1}, while the random form has an average half-width value near 160 cm^{-1}. Hence an important conclusion follows: in all the studied proteins an exchange takes place primarily in random coil parts of the molecule first and then in more ordered parts. It corresponds to the observed breaking points on the experimental curves of Figure 3, which divide unordered and regular parts of the protein molecule. Their S_r values, equal to 39% for phage T4 lysozyme and 57% for hen egg-white lysozyme, are very close to the amount of random form in these protein molecules. These points are also situated at a level near 90 cm^{-1}, which corresponds to the boundary value of amide A band half-width between unordered and ordered forms of polypeptide structures. So we estimate the value of S_r for myoglobin to be equal to about 33%. We can also conclude that the smaller the band half-width of NH-vibrations, the less is the degree

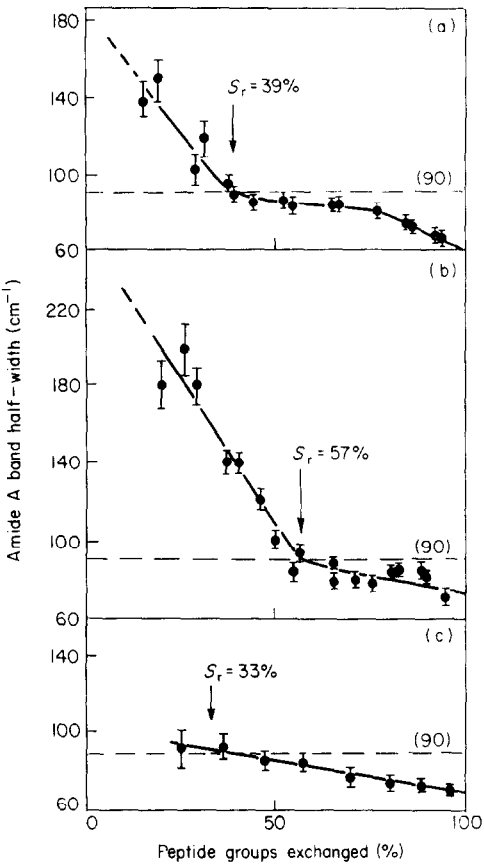

FIG. 3. The dependence of the amide A band half-width of successively exchanged peptide groups on the total amount of exchanged groups.

(a) Bacteriophage T4 lysozyme; 0·001 M-KCl (pD 4·4).

(b) Hen egg-white lysozyme; 0·001 M-KCl (pD 4·4).

(c) Sperm-whale myoglobin; 0·05 M-phosphate buffer (pD 5·6).

All preparations were solutions in D_2O. The data were obtained from several independent measurements.

of dynamic accessibility of these peptide groups for heavy water molecules or their ions.

4. Discussion

At present the deuterium exchange kinetics of proteins is accounted for by the existence of a number of regions with rate constants differing 10^6 to 10^8-fold (Hvidt & Wallevik, 1972). It is fairly difficult to classify them according to curves of exchange kinetics, although such attempts have been made (Nakanishi & Tsuboi, 1974). Our data show that such a division can be made on the basis of structural features, and the amide A band half-width, which reflects the degree of distortion of peptide hydrogen bonds, can be used in this way. We can estimate the degree of overlapping of separate classes of peptide groups with very similar values for the half-width of the amide band. Evidently this overlapping is not large, since the neighbouring experimental points on the curve were obtained in the majority of cases after a

temperature jump of 20 to 30 deg. C, which corresponds to the transition from one constant of exchange rate to another differing by an order of magnitude or more. Thus, as an approximation, the obtained curves in Figure 3 can be treated as the curves of the degree of distortion of peptide hydrogen bonds of groups being exchanged. The correctness of the conclusion is confirmed by the presence of a break on the curves at a half-width of 90 cm^{-1}, since at just such a value the exchange in unordered parts of a protein molecule is completed. Indeed, the quantity of unordered form estimated by this criterion for all the studied proteins with a known spatial structure practically coincides with that obtained by X-ray analysis.

Thus, the analysis of a change in the amide A band half-width depending on the degree of exchange permits us to consider that in globular proteins there is a distribution of parameters of peptide hydrogen bonds. In this case the more distorted the parts of the peptide structure, the more accessible they are to a water molecule or its ions. A possible degree of distortion can be estimated from the data presented by Chirgadze *et al.* (1976). The average shift of atomic co-ordinates of the main chain in the distorted α-helical structure is 0·5 Å, and the root-mean-square deviation of lengths of peptide hydrogen bonds is about 0·2 Å. Heterogeneity of the secondary structure from the geometrical point of view corresponds to the requirement of most compact packing of atoms in a protein molecule (or in the protein structure of some higher organization). This also corresponds to a lower energy level of the system. Thus, it becomes clear that the final native structure of proteins is formed due to distortion of regular fragments of their molecules. The structural distortions of the polypeptide backbone should be reflected in the position of atoms in the static spatial model of the protein molecule.

We can assume that the fragments most inaccessible to water are located in hydrophobic regions of the molecule. This does not contradict the preliminary data on localization of exchanged peptide atoms of hydrogen in a deuterated crystal of sperm-whale myoglobin (Schoenborn, 1971). Indeed, in all the cases examined peptide hydrogens belong to residues located in a hydrophobic or partially hydrophobic environment. Chirgadze & Ovsepyan (1972) have shown that conformational flexibility of the polypeptide structure is of a hydrational nature. Keeping this in mind, it is possible to assume that the less distorted fragments of the peptide structure have the least conformational flexibility.

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