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Main-chain Sorption of Water by Serum Albumin

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Infrared spectra were recorded of human serum albumin and of extensively acetylated and methylated derivatives as films. Each specimen was examined after drying as well as after equilibration with water vapour at 80 % relative humidity. After hydration an OH-stretching band of sorbed water is observed as well as changes in the Amide I and II bands and the Amide V region of the protein. These changes were similar to those reported by previous investigators for hydration of collagen and keratin. The observations seem to indicate that water, in these proteins, is sorbed to the peptide groups of the main chain rather than to polar side chains.

Pauling [1] in 1945 summarized evidence in favour of binding of water to polar side chains in several proteins, including serum albumin, and expressed the opinion that peptide carbonyl and imido groups usually have little attraction for water, due to the NH ··· O=C hydrogen bonding. Several observations have been interpreted accordingly, including those of Brey *et al.* in 1968 [2,3] who studied the nuclear magnetic resonance of water sorbed on serum albumin. On the other hand, keratin and collagen seem to bind water to the main chain, as evidenced from infrared spectroscopy by Bendit [4] and by Susi *et al.*,[5] respectively. Malcolm [6] has examined the infrared spectrum and dichroism of water adsorbed on poly-alanine, in which polar side chains are absent, and has found that the water molecules are situated in specific orientation with respect to the helical polymer, probably with a weak hydrogen bond to peptide oxygen and with a strong dipole interaction between water and peptide. Water sorbed on γ-ethyl and γ-methyl esters of poly-glutamate, according to Malcolm, likewise gives a dichroic OH-stretching band. The water is bound to the main chain and possibly to sidechain carbonyl as well.

In the present paper infrared spectra of hydration are reported for human serum albumin and for two derivatives, one of which is extensively methylated in the carboxyl side chains, and another which is acetylated in lysyl amino groups. The aim is an investigation of the binding sites of water.

EXPERIMENTAL

Human serum albumin (Kabi) was deionized in a mixed bed ion exchange column and lyophilized. The natural content of fatty acids, about 0.5 mol per mol protein, was not removed. The preparation contains about 7 % dimer and trimer albumin. The mol weight was taken as 66 000. The number of aminoacyl residues is about 570, and Stoke's radius of the monomer is 3.6 nm.[7]

Methylated albumin was prepared by reacting albumin (1 g) with 60 ml methanol, 0.1 M with respect to hydrochloric acid, for two days at room temperature. The mixture was then neutralized, dialysed, and lyophilized. Titration showed that 70 carboxyl groups per mol albumin corresponding to 73 % were esterified. The Stoke's radius could not be estimated due to low solubility. The peptide chain is largely unfolded, according to Sun.[8]

Acetylated albumin was prepared as described previously.[9] 56 amino groups per mol albumin corresponding to 96 % were acetylated. The Stoke's radius was 4.9 nm, determined by Sephadex gel-filtration in a 0.1 M phosphate buffer pH 7.4 at 5°C. This radius is significantly higher than that of the native protein, indicating unfolding of the acetylated molecule.

Films of protein were prepared by spreading 15 μl of a 1 % solution in water over each of four thallium bromide iodide (KRS-5) slabs until spontaneous drying, whereby a film of 0.16 mg/cm^2 was formed on each slab. Two of these were placed over strong sulphuric acid (93 – 94 %) at atmospheric pressure and room temperature (23°C) overnight. These moderately dried films were placed in the reference beam, as described below. Two slabs were placed over diluted sulphuric acid overnight for equilibration at a predetermined relative humidity.[10] Before opening the container, each pair of slabs were placed on either side of a rubber spacer, with the protein carrying aspects facing each other. The thickness of the spacer, 1 mm, was chosen large enough to avoid interference fringes in the spectrum, and small enough to prevent evaporation of appreciable amounts of water from the films when the temperature rose during recording. The temperature in the sample compartment was around 35°C. Each set of films, sample and reference, was moved perpendicularly to the beam by means of adjustment screws until equal protein film thickness was obtained, as judged from the spectrum. It was presumed that the CH_3 and CH_2 stretching bands at 2900 – 3000 cm^{-1} were not influenced by hydration. Neutralization of these bands were taken to indicate proper positioning. The choice of these bands for equalizing the sample and reference protein thickness was to some extent arbitrary. It was found, however, as seen from Figs. 1 – 2, that the Amide A band was neutralized as well, and the spectrum in the Amide I and II region could be interpreted as due to changes on hydration, thus confirming the constancy of the bands at 2900 – 3000 cm^{-1}.

Cracking of the dry films, signalled by discontinuities of the record, sometimes necessitated repeated experiments.

Spectra were recorded on a Beckman IR 20A infrared spectrophotometer from 4000 to 250 cm^{-1}. Slow scanning with carefully balanced gain, damping, and slit width was necessary to obtain reproducible details in the difference spectra. The time taken for each record was 4 to 16 h.

The amount of water sorbed was determined on a Cahn Gram Electrobalance. About 15 mg of the lyophilized protein were placed on one pan and a known relative humidity (r.h.) was produced by a dish of diluted sulphuric acid placed in the chamber. Constant weight was obtained in 24 h at relative humidities between 10 and 70 %. Measurements were begun at 10 % r.h., each day shifting to a higher value. The original weight at 10 % r.h. could be reproduced at the end. 10 % r.h. was chosen as a basis since it is difficult to obtain reproducible weights by more exhaustive drying. The temperature was 23°C ± 1°C.

RESULTS

Fig. 1 shows the infrared spectrum of a dried albumin film, the difference spectrum of hydration at 80 % r.h., the calculated spectrum of the wet film, obtained by adding these, and finally, the spectrum of liquid water, approximately in the same amount as found in the wet film. Equilibration at 20 % r.h.

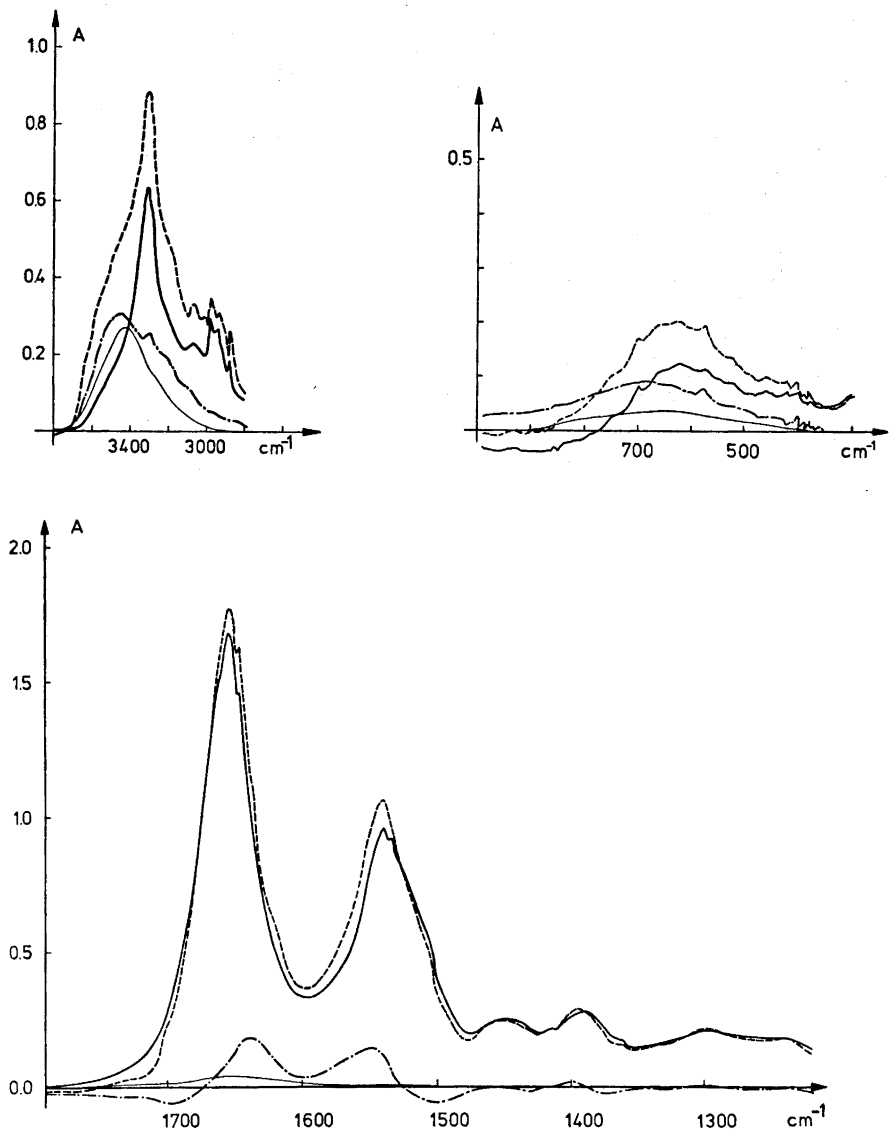

Fig. 1 a–c. ——— Infrared spectrum of human serum albumin (HSA) film, moderately dried over concentrated sulphuric acid. — · — Difference spectrum of moist HSA, 80 % r.h., against dried HSA. - - - Spectrum of moist albumin, obtained as the sum of the above spectra. ——— Spectrum of water, approximately same amount as in the moist albumin films. Ordinates, absorbance.

resulted in a difference spectrum (not shown) qualitatively similar to that of 80 % r.h. but with proportionately lower amplitudes. In repeated experiments the following spectral changes on hydration were reproducible:

In the 3 μ region the difference spectrum is rather similar to that of liquid water, except that two separate maxima are seen, at 3460 and 3300 cm^{-1}, while the principal maxima of liquid water at 3490 and 3280 [11] are registered as one skew peak.

In the 6 μ region the Amide I band is shifted slightly towards lower frequencies and the Amide II frequency is increased; both bands show higher intensities after hydration. The band at 1400 cm^{-1} (side chain carboxylate) is shifted towards higher frequencies.

In the 15 μ region is seen a shift of the Amide V band to higher frequencies and an increase of intensity. The spectral changes are larger than those which could be caused by a similar amount of liquid water alone.

Difference spectra of hydration of two albumin derivatives are shown in Fig. 2. These display the same main features as does the spectrum of native albumin, namely the double water peak and the changes of Amide I, II, and V bands. After extensive methylation of side-chain carboxyl groups no hydra-

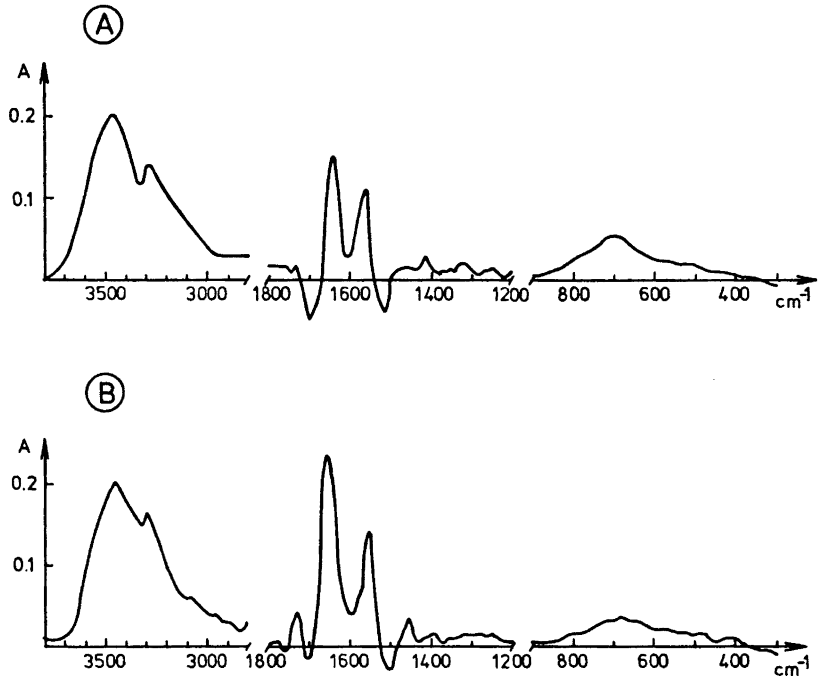

Fig. 2. Infrared difference spectra of hydration of albumin derivatives. A film of each derivative was equilibrated at 80 % r.h., and measured against a dried film of the same substance. A, all ε-amino groups acetylated. B, most carboxyl groups methylated. Ordinates, absorbance.

tion shift is observed at 1400 cm^{-1} while a change is seen at 1750 cm^{-1}, presumably a decrease of the non-hydrogen-bonded C=O absorption.

Albumin acetylated in lysyl NH_2 groups shows a hydration difference spectrum almost identical with the difference spectrum of hydration of native albumin. The shift observed at 1400 cm^{-1} for native albumin has almost disappeared in the acetylated sample in agreement with the fact that side-chain carboxyl groups are protonized in the isoelectric state.

The amounts of water sorbed to human serum albumin and to the two derivatives are depicted in Fig. 3. Approximately 100 mol H_2O could be removed

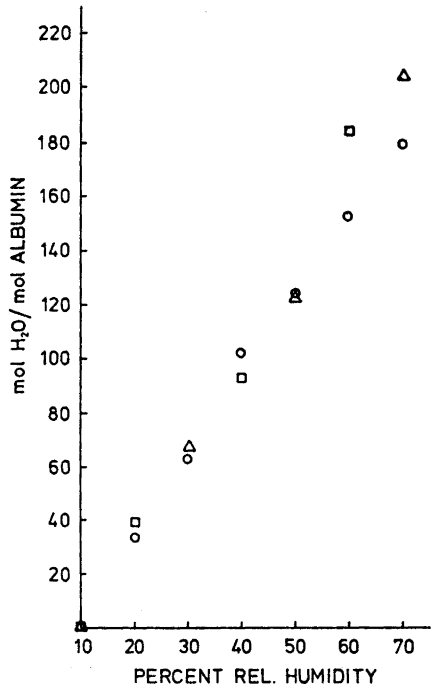

Fig. 3. Amount of sorbed water as a function of relative humidity. O, human serum albumin. △, HSA, most carboxyl groups methylated. □, HSA, all ε-amino groups acetylated.

from each mol protein, equilibrated at 10 % r.h., by drying over conc. sulphuric acid. This amount is similar in the native albumin and the two derivatives. It is noted from Fig. 3 that the amounts of water sorbed in the range 10 – 70 % r.h. are equal in the three substances. Extensive methylation of carboxyl groups or acetylation of amino groups, which are the main polar groups of the side chains, does not influence binding of water to a measurable extent.

DISCUSSION

The main infrared spectral changes due to water sorption to human serum albumin involve the principal main-chain frequencies, Amide I, II, and V. This seems to indicate that water is bound to the peptide groups of the main chain.

Binding of water exclusively to polar side-chain residues on the surface of the protein molecule could not explain the shifts observed of the main-chain bands.

After extensive chemical alterations of the polar side chains the main features of the hydration difference spectra remain unchanged. This is found after esterification of most of the carboxyl groups as well as after acetylation of all amino groups. This supports the above conclusion, that water is sorbed to the main chain.

The amount of water sorbed to the protein is not changed by these chemical modifications of the polar side-chain residues. This adds further evidence against binding of water solely to these groups.

On the other hand, it seems that carboxylate groups, besides the main chain, are involved in the binding of water molecules, as evidenced by the shift of the band at 1400 cm^{-1} by hydration of native albumin. This shift is not observed on hydration of albumin in which the carboxyl groups are esterified. In the albumin with acetylated amino groups the hydration shift at 1400 cm^{-1} is nearly absent. In this derived protein most of the carboxyl groups are protonized since 56 ammonium groups of the native albumin have lost their positive charge. Twenty-three quanidinium groups remain positively charged and a corresponding number of COO^- are unprotonized. This agrees well with the finding of a slight hydration shift of the carboxylate band at 1400 cm^{-1} if it is presumed that water may bind to these groups.

The albumin derivative with methylated carboxyl groups shows a decrease of infrared absorption at 1750 cm^{-1} on hydration. A number of non-hydrogen-bonded CO groups are probably present in this substance and contributes to a peak at 1750 cm^{-1} seen in the infrared spectrum. On hydration hydrogen bonds are probably established to these groups and the peak at 1750 cm^{-1} disappears into the large Amide I band. This probably explains the negative peak in the difference spectrum of hydration and indicates a role of the ester carbonyl in binding of water.

The carboxylate groups of the native albumin and the ester groups of the methylated derivative may contribute to binding of water by establishing additional bonds to water molecules already sorbed to the main chain or may bind water which is not bonded to peptide groups. In the latter event the amount of water sorbed would be influenced by the chemical modification if the binding affinity is changed. As seen from Fig. 3 the changes expected would hardly be perceptible and it is thus not possible to conclude whether water molecules may be bound to carboxylate and ester residues independently or with bonds to peptide groups as well. At any rate sorption of water to the main chain quantitatively out-weighs binding to polar side chains to a considerable extent.

The peptide chain is largely unfolded in both of the derived albumin preparations. Acetylation thus caused an increase of the Stoke's radius from 3.6 to 4.9 nm. The viscosity of methylated albumin has been measured by Sun [8] and likewise indicates unfolding to a considerable extent. It is interesting to note that the unfolded peptide chain shows the same changes of infrared spectrum on hydration as seen in the native protein. Also the amount of water sorbed remains the same after unfolding of the peptide chain. Loosely bound water molecules would thus appear to form an integral part of the native peptide structure of the albumin molecule.

Bendit [4] has recorded hydration difference spectra for keratin. These show the same main features, as reported above for serum albumin, *i.e.* a double maximum in the OH stretching region, similar to that of liquid water, lowering of Amide I and increase of Amide II frequencies with increased intensities. These changes are seen in α-keratin, in supercontracted "randomized" α-keratin and in β-keratin. The spectra reported by Bendit do not include the Amide III and V bands.

Susi *et al.*[5] have examined infrared spectra of collagen with varying amounts of bound water and have found similar changes in the OH-stretching and Amide I and II regions, as for albumin and keratin. The Amide V bond of collagen is shifted to higher frequencies with increased intensity, as in albumin. In addition the authors report a shift of CH-bending frequency at 1450, ascribed to hydrogen bonding to CH. This is not seen in keratin,[4] nor in albumin. The Amide III band of collagen at 1232 cm^{-1} is increased in frequency and intensity on hydration. A similar change is not seen in the albumin spectra. The Amide III band of an albumin, however, has a low intensity, and relative changes, similar to those in collagen, would not be perceptible.

The infrared spectrum of procollagen [12] shows similar changes on hydration.

Poly-lysine hydrochloride [13] and sodium poly-glutamate [14] exhibit patterns of infrared spectral changes on hydration, different from those of albumin, keratin, and collagen. These changes are due to $\alpha \rightarrow \beta$ transitions, caused by the electric charges of the side chains. Similar transitions are not seen in the proteins mentioned.

Buontempo *et al.*[15] recently reported that the difference spectrum of water bound to lysozyme and to bovine serum albumin shows two maxima, one at 3450 cm^{-1} and another somewhat below 3300 cm^{-1}. These are due to rather loosely bound water, similarly to the water dealt with in the present paper. Buontempo *et al.* have found that, in addition to this, some more tightly bound water may be removed by prolonged evacuation. This water shows a single, broad maximum at 3260 cm^{-1} when examined near liquid nitrogen temperature. Infrared spectral changes at Amide I, II, and V frequencies have not been reported for sorption of this tightly bound water.

In conclusion, a common pattern of changes of infrared spectra on hydration appears to exist in several very different proteins so far examined, *viz.* keratin in α-, β-, and amorphous conformation, collagen (triple helix), and in serum albumin (globular with partial α-helix structure) in the native state as well as after chemical modifications of polar side chains causing unfolding. This pattern can be described as follows:

(1) The OH stretching band of sorbed water shows two separate peaks, in the vicinity of those of fluid water (3490 and 3280 cm^{-1}), the former with the higher intensity.

(2) The Amide A band near 3300 cm^{-1} is unchanged. (Bendit [4] interprets the two OH stretching bands as one, modulated by a slight decrease of the Amide A frequency.)

(3) A small decrease of the Amide I frequency and an increase of the Amide II frequency. The ratio of these shifts is approximately 1:4. Increased intensity of both bands.

(4) Increased frequency and intensity of the Amide V band.

These changes are seen on sorption of relatively loosely bound water from a few per cent relative humidity to about 60 – 90 %. The amount of water bound is less than one molecule per peptide group in albumin and collagen, and less than two in keratin.

The main peptide chain seems to be involved in the binding of water in all these cases. Hydrogen bonding to peptide C = O groups may explain the changes of the Amide I, II and V bands.[4–6] The appearance of two separate peaks in the OH stretching region indicates that a water molecule is bound in a specific position in relation to the carbonyl group. This is supported by the findings of Malcolm [6] who studied infrared spectra and dichroism of water sorbed on α-helical synthetic polypeptides. The findings are consistent with binding of a water molecule through a hydrogen bond to peptide oxygen together with a strong interaction between the dipoles of water and peptide. The water molecule is thus located in a specific position which is tentatively described by Malcolm.

Brey *et al.*[2,3] have measured spin-spin relaxation times of protons of water sorbed on bovine serum albumin as a function of water coverage and temperature. They found results which are in agreement with binding of water to polar side chains. These authors emphasize a discontinuity of the NMR line width at 160 mg H_2O per gram protein. This corresponds precisely to one water molecule per peptide unit of the main chain. It may be possible to reinterpret the findings of Brey *et al.* in the light of water sorption to the peptide backbone.

Other residues, including those of side chains, take part in the binding of water, either by establishing additional hydrogen bonds to the H_2O molecules already bound to C = O, or by binding more water. This explains the variable amount sorbed to different proteins (Bull [16]). Polymers containing amide groups but without polar side chains, such as poly-alanine [6] and nylon,[16] have a low affinity for water. Even nylon may, however, bind nearly one molecule of H_2O per amide bond by exposure to saturated water vapour.[16]

Since proteins are synthetized in an aqueous surrounding it is likely that water forms an integral part of the basic peptide structure.

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