STRUCTURAL CHANGES OF MET-HAEMOGLOBIN BY DEHYDRATION

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1. Introduction

As was emphasized by Kuntz and Kautzmann in a recent review [1], the interpretation of absorption isotherms for water-uptake by dried proteins is difficult, for lack of experimental data about the structure of partially or fully dehydrated proteins. They suggest that the shape of absorption isotherms and their hysteresis may be determined by a 'substantial refolding of the polypeptide chains and rearrangements of side-chains in order to reduce the size of voids created on drying a protein'.

To prove this assumption, we have done parallel investigations on the influence of dehydration on the CD-spectra (190–240 nm) and the absorption spectra (300–700 nm) of Met-Hb* prepared as films. Our results show a complicated, at least biphasic, dependence of the Met-Hb structure on dehydration. Removal of the first amount of the more weakly bound water (from 100–70% r.h.) produced a hemichrome form as indicated by absorption spectra; only minor changes are induced in the secondary structure as indicated by CD-spectra.

Withdrawal of the last amount of the more strongly bound water (from 70–0% r.h.) diminished the helix content of Met-Hb by 25–30%. The conformation of the dried protein corresponds to the solution and film structure of a Met-Hb complex with 15 mol SDS/mol subunit.

The results have shown that withdrawal of any water, even that weakly bound, results in conformational changes of Met-Hb. The data described in this paper require a more intricate interpretation of differences in dehydration of Met-Hb and deoxy-Hb than suggested by Killion et al. [2,3] through differences in structures of liganded and non-liganded haemoglobins after crystallographic results.

2. Materials and methods

Met-Hb was obtained by oxidation of human HbO_2 with K_3 ($Fe(CN)_6$) followed by gel-filtration through a Sephadex G-25 column. Films of Met-Hb were prepared on CaF_2-windows by evaporation of protein solutions that had been dialyzed several times against bidistilled water. The pH-values were adjusted by titration of the protein solutions with 0.1 N NaOH and 0.1 N HCl, respectively. SDS was twice recrystallized from ethanol.

A special film technique [4,5] permitting continuous variation of r.h. from 100–0% was applied for the measurements of the CD- and absorption-spectra. All spectra were recorded at 25°C. Circular dichroism measurements were performed using a Roussel-Juan-Dichrograph CD 185. Solutions were measured in cuvettes with 0.1 cm path-length (cf.

*Abbreviations: Met-Hb, Met-haemoglobin; SDS, Sodium dodecyl sulphate; r.h., relative humidity

figs.2 and 3). The θ-values of the native Met-Hb in solution were calculated to be 19 200 (pH 6.0) and 19 800 deg.cm^2.dmol^{-1} (pH 8.5) at 222 nm. The CD-spectra of films (at highest r.h., thickness approx. 0.5 μm) show nearly identical shapes with the solution curves and were normalized to agree in the ΔD-values at 222 nm. Electronic absorption spectra were obtained on a spectrophotometer UV/ VIS Specord (VEB Carl Zeiss, Jena).

3. Results

Detailed investigations of the influence of dehydration on Met-Hb films were done at pH 6.0, where Met-Hb in solution preferentially exists in the high-spin aquo-form. At highest r.h. (99.7%) the spectra of Met-Hb films are practically identical in shape with those of the native protein in solution, as shown in fig. 1. Decreasing the r.h. by only 1–2% causes slow changes of spectra which are accelerated and more extensive if the r.h. is decreased quickly by 10–15%.

At 70% r.h. the spectra completely correspond to low spin hemichrome with maxima at 412 nm, 536 nm and a shoulder at 565 nm. All spectral changes are fully reversed by rehydration. Isosbestic points between the spectra from native Met-Hb and the hemichrome structure are at 478, 516, 595 and 660 nm. Further dehydration below 70% r.h. causes

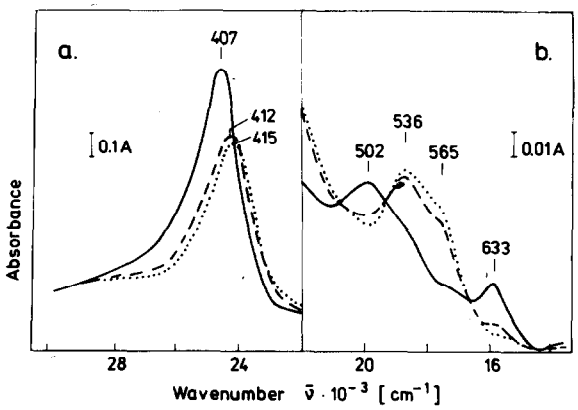

Fig.1. Absorbance spectra of Met-Hb films (pH 6.0) at different % r.h. (——) 99.7% r.h., (– – –) 71.0% r.h., (. . . . .) 0% r.h.; indicated wavelengths are in nm.

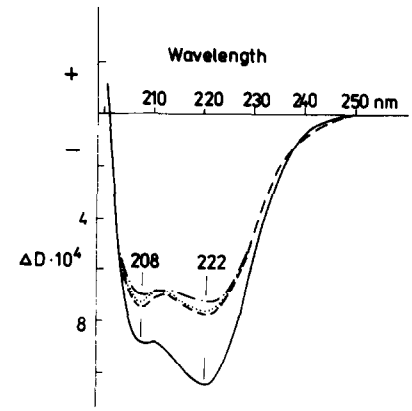

Fig.2. CD-Spectra of Met-Hb (pH 6.0) in solution and as films at different % r.h. (see Materials and methods). Solution (– · – · –), 0.97 × 10^{-5} M; Films, (——) 99.7% r.h., (– – –) 71.0% r.h., (. . . . .) 0% r.h.

only minor spectral changes, and at 0% r.h. there is a small increase in scattering caused by destruction of the film, that is apparent visually.

The results indicate that the main structural changes of the haem pocket of Met-Hb occur in the range 99.7 – 70% r.h. An entirely different situation exists for the CD-spectra in the region of peptide absorption. Decreasing the r.h. from 99.7 – 70% induces, at pH 6.0, only a negligible decrease of ellipticity in the 190–240 nm range. But the ellipticity of the 222 nm band is decreased through further dehydration up to 0% r.h. by about 25% (fig.2). Contrary to the changes in the haem pocked the partial destruction of secondary structure (decrease of the helix content) mainly occurs on dehydration in the range 70 – 0% r.h. These changes are similar to changes induced by addition of relatively small amounts of SDS (15 mol SDS/mol subunit) to alkaline solutions of Met-Hb. The ellipticity of the 222 nm band decreases likewise by 25–30% and equals the 208 nm band in intensity (fig.3).

The absorption spectra of this SDS-complex in solution show the characteristic hemichrome bands with a small red-shift of the Soret band to 416 nm and a β-band at 540 nm. Suprisingly there exists no marked difference in the absorption and CD-spectra between the SDS–hemichrome in solution, highly hydrated and dehydrated films. (Only at 0% r.h. does the 222 nm CD-band decrease by about 10% and

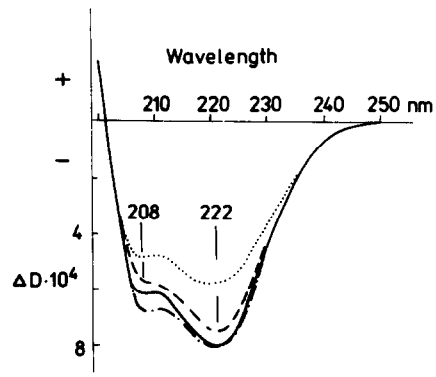

Fig.3. CD-Spectra of Met-Hb (pH 8.5) by addition of 15 mol SDS/mol subunit (see Materials and methods). Solutions, 1.18×10^{-5} M (——) without SDS, (– – –) with SDS. Films with SDS (. . . . . .) 99.7% and 71% r.h., (– · – · –) 0% r.h.

there is a small decrease in overall intensity of absorption bands because of film desctruction.) Obviously, structures similar to those of extensively dried proteins may be produced also in solution by addition of small amounts of detergents and are largely independent of hydration.

We have found similar changes in absorption and CD-spectra by dehydration of Met-Hb films also at other pH-values in the pH-range 5.1–9.8.

4. Discussion

The main changes in the secondary structure of Met-Hb occur by dehydration in the range 70–0% r.h. (the range of hysteresis of the absorption isotherms). Water sorption measurements in this range show for films at 24°C [6], as well as lyophilized powders at 20°C [7], a loss of nearly 0.15 g water/g protein, that means a solvation-shell somewhat higher than 2 BET-monolayers coverage. The substantial disturbance of the protein conformation as a consequence of the removal of this 'non-freezable' water (so classified from calorimetric and PMR data cited in [1]) leads to changes of the dielectric properties [7] and an alteration in the partial volume (cited in [1]) of Met-Hb and is connected with the appearance of the kink in the absorption isotherms [2]. Obviously, the desorption of this more strongly-bound water cannot be described only as a passive removal of water molecules from hydrated charged and polar groups because there is involved also a contribution arising from the conformational rearrangements of the polypeptide chains.

It is important to remember that in this range of absorption-isotherms (below 70% r.h.) the differences in hydration between Met-Hb on the one hand and deoxy-Hb and liganded low spin forms of Met-Hb on the other are maintained [2,3]. In fact, as supported by our investigations, these differences are related to the differences between hemichrome and hemochrome because the transition from deoxy-Hb to hemochrome takes place, according to Haurowitz [6], on hydration of 0.12 g water/g Hb or 50–60% r.h. at 24°C. Thus, we conclude that the differences in hydration behaviour between liganded and non-liganded Hb-forms may be linked not with the different size of the central cavity as suggested by Killion et al. [2,3] but reflect differences in the stability of Met-Hb and all other forms of Hb and/or different structures in the hemichrome and hemochrome form, respectively.

Conformational changes are observed also in the range of higher r.h. (above 60–70%) where the hydration depends more strongly on changes of r.h. From hydrodynamic and PMR data (quoted in [1]) a hydration of Hb in solution of 0.4–0.5 g water/g Hb was estimated. Taking this data for films at r.h. near 100% the calculated amount of the more weakly bound water removed by the formation of the hemichrome comes to 0.35–0.25 g water/g Hb. Even if we find no essential changes of CD-spectra in this dehydration step, a subtle and functionally important perturbation in the protein structure could have taken place which is not reflected in the secondary structure. (Compare, for example, the latest X-ray results for Met-Hb and CN-Met-Hb [8] and data for other proteins [9].) It was proposed [10] that in such reversible hemichromes the sixth coordination position is occupied by the distal histidine E 7. Hemichromes with analogous absorption spectra maxima (cf. fig.1) are formed also in solution by addition of neutral salts [11], organic solvents [12] and drugs [13], spontaneously from isolated subunits of Met-Hb [14–16], by freezing Met-Hb solutions [17], by dissolution of Met-Hb in glycerol [18,19] and also in the process of heat-denaturation in vitro [15] and destruction of Hb in vivo [13]. Anusiem et al. [19] have proposed that, at pH 6.0, hemichrome is in

equilibrium with the native aquo-Met-Hb in water solutions.

The formation of the hemichrome may arise both from a decrease of the water content and from distortion of contacts between the subunits of Met-Hb. The static three-dimensional structure of hemichrome may preserve many features of the native structure and apparently this new structure has a considerable specificity ('quasi-denatured' as named by Brausse et al. [7]). Moreover, several hemichromes are characterized by high conformational motility as shown by means of sensitive methods of $^{1}H-^{2}H$ exchange and of proteolytic degradation [5].

Since the water absorption isotherms of Hb [6,7] are similar to the isotherms of other proteins, comparable structural changes by dehydration may be a general property of globular proteins. However, their concrete manifestation must strongly depend on individual structural stability of the protein in each case. Thus, according to our preliminary data the decrease in helix content and disturbance in the struc ture of the haem pocket of Met-myoglobin practically take place simultaneously and at significantly lower r.h. (below 40%) than with Met-Hb.

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References

[1] Kuntz, J. D., Jr. and Kautzmann, W. (1974) Advan-Protein Chem. 28, 239–345.
[2] Killion, P. J., Reyerson, L. H. and Cameron, B. F. (1970) J. Colloid. Interface Sci. 34, 495–504.
[3] Killion, P. J. and Cameron, B. F. (1974) Biopolymers 13, 1653–1659.
[4] Böhm, S., Krumbiegel, J. and Billwitz, H. (1974) Eur. J. Biochem. 41, 617–623.
[5] Krumbiegel, J., Böhm, S., Pommerening, K., Wendel, I. and Wetzel, R. (1976) Stud. Biophys. 59, 93–100.
[6] Haurowitz, F. (1951) J. Biol. Chem. 193, 443–452.
[7] Brausse, G., Mayer, A., Nedetzka, T., Schlecht, P. and Vogel, H. (1968) J. Phys. Chem. 72, 3098–3105.
[8] Deatherage, J. F., Loe, R. S., Anderson, C. M. and Moffat, K. (1976) J. Mol. Biol. 104, 687–706.
[9] Fletterick, R. J., Sygysch, J., Murray, N., Madson, N. B. and Johnson, L. N. (1976) J. Mol. Biol. 103, 1–13.
[10] Peisach, J., Blumberg, W. E. and Adler, A. (1973) Ann. NY Acad. Sci. 206, 310–327.
[11] Scheler, W., Graf, W. and Scheler, J. (1963) Acta Biol. Med. Germ. 11, 463–479.
[12] Bucher, D. J. and Brown, W. D. (1971) Biochemistry 10, 4239–4246.
[13] Peisach, J., Blumberg, W. E. and Rachmilowitz, E. A. (1975) Biochim. Biophys. Acta 393, 404–418.
[14] Rachmilowitz, E. A., Peisach, J. and Blumberg, W. E. (1971) J. Biol. Chem. 246, 3356–3366.
[15] Abaturov, L. V., Ushakova, M. M., Molchanova, T. P., Jakobashvili, N. N., Zhdanova, K. J. and Dzhinoriya, K. Sh. (1975) VII. Int. Symp. Struktur und Funktion der Erythrozyten, pp. 129–133, Akademie-Verlag, Berlin.
[16] Bucci, E. and Fronticelli, C. (1971) Biochim. Biophys. Acta 243, 170–177.
[17] Iuzuka, T. and Kotani, M. (1969) Biochim. Biophys. Acta 194, 351–363.
[18] Scheler, W., Schoffa, G. and Jung, F. (1957) Acta Biol. Med. Germ. 11, 463–479.
[19] Anusiem, A. C. J., Beetlestone, J. G., Kushimo, J. B. and Oshodi, A. A. (1976) Arch. Biochem. Biophys. 175, 138–143.