ACTA CHEMICA SCANDINAVICA 17 (1963) S 216–S 221

Effect of Electrolytes on the Absorption Spectrum of Alkaline Ferriheme

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Spectral changes observed in the region 270–650 mμ upon addition of various electrolytes to $10^{-3} - 10^{-5}$ M alkaline ferriheme (ferriprotoporphyrin IX) are mainly characterized by the appearance of a broad band around 580 mμ, a minimum near 540 mμ and by a considerable diminution of the Soret band maximum at 385 mμ. In the visible range, these changes were found to depend on both the ionic strength of the added electrolyte and the total ferriheme concentration. In the system 5×10^{-5} M ferriheme – 1.9 M NaCl, the ratio $\varepsilon_{580}/\varepsilon_{540}$ decreased significantly from pH 9 to 5.5 indicating a transition with an estimated midpoint of pH 7.1 at 27°C. Both spectral and sedimentation-diffusion data suggest that ferriheme molecules, at high ionic strength, form micelles of considerable size in which optical interactions may occur. This aggregation is considered to be favored by screening of electrostatic interactions between porphyrin carboxylates and also by changes in the structure of water around the hydrophobic regions of the protoporphyrin.

The absorption spectra of the prosthetic group iron-protoporphyrin IX as they appear in various hemoproteins and their derivatives have been classified by Theorell[1] many years ago on the basis of extensive research on these substances. A recent treatment of the absorption bands of ferric porphyrin complexes[2] suggests two charge-transfer bands to high-spin ferric ions and three porphyrin absorption bands which appear in low-spin complexes.

A spectral type not found in native systems has been observed in ferriheme solutions of pH > 9 containing high concentrations of pyridine (1 – 6 M)[3–6] and, more recently, in the presence of random or highly ionized polybases such as poly-L-lysine at pH 8 – 9[7] or poly-4-vinylpiperidine[8]. These "green complexes" appeared whenever the regular helical structure of poly-L-lysine-ferrihemochromes seemed to be disturbed *e. g.* by ionization, copolymerization or increase in temperature[8]. It has now been found that simple electrolytes, at sufficiently high concentrations, also caused the appearance of analogous spectra. The present investigation is concerned with the formation of the "green complexes" produced by salts, in particular as measured by the visible-range absorption. These complexes probably constitute higher aggregates. According to the con-

ditions for their formation, similar aggregates will not be expected to be produced in native proteins under normal conditions.

EXPERIMENTAL

Materials. Sigma Bovine Hemin (2 × recrystallized) was again recrystallized. Salts used were analytical-grade.

Spectra. Beckman DU and Zeiss PMQ II spectrophotometers were used. Constant temperature (usually 26–27°C) was maintained by circulation around the cell compartment of water from a temperature-controlled bath.

Sedimentation and diffusion were measured in a Spinco Model E Ultracentrifuge using Schlieren optics with Kodak plates type 103a-E and a red Ilford filter 205.

Stability and reproducibility. The absorbancy of three different solutions of 0.4, 0.7 and 2 M NaCl, respectively, all containing 5.1×10^{-5} M ferriheme at pH 10.5 and 26°C, was checked at 385, 540 and 580 mμ at constant time intervals up to 24 h and in one case (2.0 M NaCl) up to one week. Maximal changes in ε were of the order of 10 % but in most cases the deviations were smaller. The Soret-bandabsorption was generally less stable with time than the visible-range absorption. Parallel preparations of identical composition occasionally disagreed by 5 – 10 %, in particular at pH $\approx$ 6 which is close to the precipitation limit of the ferriheme-salt system (Fig. 4). CO_2 was not excluded rigorously from the solutions since the effect of Na_2CO_3, which could be formed by CO_2-absorption from the air, was considered negligible.

The light absorption of solutions diluted from higher concentration was in reasonable agreement with values obtained directly, though in some cases there were indications of partial irreversibility. The order of mixing of salt and ferriheme solutions had no effect on the spectrum. ε_mM is the decadic millimolar extinction coefficient in $mM^{-1} \times cm^{-1}$ calculated per 1 Fe. Concentrations of polymer and ferriheme are in monomolar units.

RESULTS AND DISCUSSION

The effect of 1.9 M NaCl on the spectrum of alkaline ferriheme in the range 270–650 mμ is given by curves 1 and 2 of Fig. 1. Under the conditions of Fig. 1,

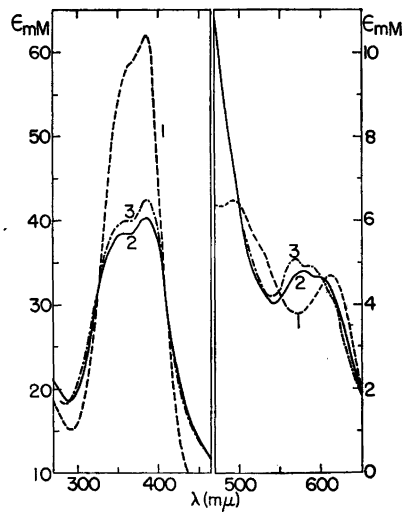

Fig. 1. Absorption spectra at 26°C. Curve 1: Ferriheme 5.1×10^{-5} M; pH 11. Curve 2: Ferriheme 5.1×10^{-5} M; NaCl 1.9 M; pH 11. Curve 3: Ferriheme 5.1×10^{-5} M; poly-L-lysine 5.0×10^{-4} M; pH 8.9.

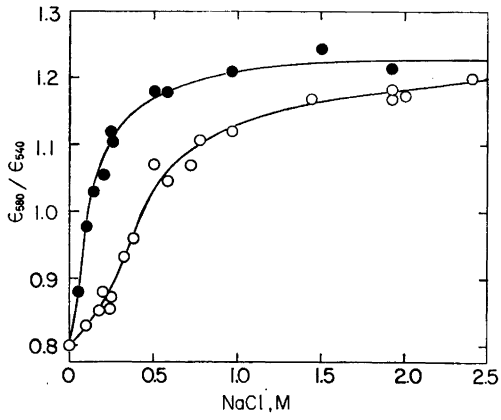

Fig. 2.

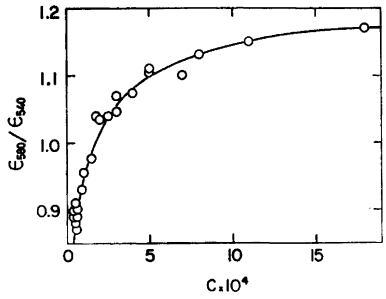

Fig. 3.

Fig. 2. Dependence of the ratio $\varepsilon_{580}/\varepsilon_{540}$ on salt concentration at 27 ± 1°C. Open Circles: Total ferriheme 5×10^{-5} M; pH 11. Dots: Total ferriheme 5×10^{-4} M; pH 11 – 12.

Fig. 3. Dependence of the ratio $\varepsilon_{580}/\varepsilon_{540}$ on the total ferriheme concentration at constant NaCl concentration (0.25 M), 27 ± 2°C and pH 11.

the Soret peak at 385 mμ is diminished to $\varepsilon_{mM} = 41$ in 1.9 M NaCl. An adjacent and pronounced shoulder near 360 mμ also decreases. In the visible range, the main band maximum is at 580 mμ ($\varepsilon_{mM} = 5$) with a shoulder near 600 mμ. Minima are at 540–545 and 290 mμ, respectively. The regular ferriheme peak near 490 mμ is absent. In spectra intermediate between curves 1 and 2, isosbestic points were not always well defined. The spectrum of the green poly-L-lysine complex at pH 9 which is added for comparison (curve 3) is very similar in the UV-region to the system ferriheme-1.9 M salt with a slight shift to the red (max. at 385–390 mμ). In the visible range, the bands appear shifted by about 10 mμ to the blue (570 and 590 mμ) relative to curve 2. The shoulder at 590–600 mμ persists at excess electrolyte or polypeptide and is therefore considered to constitute a band belonging to the green complexes. For characterization of the ferriheme-salt system the ratio $\varepsilon_{580}/\varepsilon_{540}$ is used below since it was found experimentally to give more reproducible values than either ε_{580} or ε_{385}. This may be due to optical effects occurring in systems close to precipitation.

According to Fig. 2, the formation of the green species induced by salt depends on both the salt and ferriheme concentrations. At higher ferriheme concentrations less electrolyte is required to produce an equal spectral effect. From the levelling off of the upper curve at an absorption ratio of about 1.2 it appears that 2 M NaCl converts most of the 5×10^{-5} M ferriheme into the green form. Fig. 3 demonstrates the effect of ferriheme concentration at a single salt concentration.

The dependence of the ratio $\varepsilon_{580}/\varepsilon_{540}$ on pH (conditions of Fig. 4) cannot be followed to pH $\langle$ 5.5 because of immediate coagulation of ferriheme under these conditions. This makes it difficult to estimate the midpoint of transition. Assuming $\varepsilon_{580}/\varepsilon_{540}$ to remain constant between pH 5.5 – 6.0 (between pH 10 – 13, $\varepsilon_{580}/\varepsilon_{540}$ is practically independent of pH), this pK is 7.1 ± 0.15 at 27°C. Similar

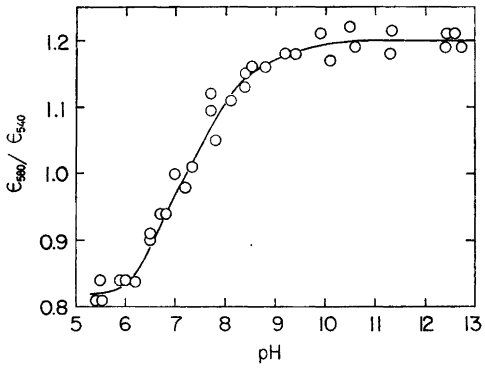

Fig. 4. pH-dependence of the spectral ratios in the presence of 1.9 M NaCl. Ferriheme 5.1×10^{-5} M; temperature 27 ± 1°C. In most experiments 0.01 M NaH_2PO_4 was included.

behavior with pH was observed by us for ferriheme in the absence of external salt. (Kajita *et al.*[9] found a p*K* of 7.3 at 20 – 22°C). Shack and Clark[6] found a p*K* of 7.4 – 7.6 for ferriheme and attributed the change to neutralization of an OH^- attached to the central iron. This could also explain the pH-dependence of the ferriheme-salt system which is fairly close to a simple titration curve. The state of aggregation of this neutralized ferriheme was not investigated. Reduction of 5×10^{-5} M ferriheme in 2 M NaCl at both pH 10 and 6 by $Na_2S_2O_4$ produced the typical ferroheme spectrum observed in the absence of salt. (The absorption was, however, lower in salt solution.) Again, this indicates participation of the OH^- in forming the green species.

Above pH 8 spectral changes with temperature of ferriheme-NaCl remained within the limits of experimental error in the range 5–40°C.

The effect of salts other than NaCl on ferriheme was also tested. At pH 11, 0.25 M Na_2SO_4 or K_2CO_3 and 0.6 M NaCl caused similar spectral effects in the visible range, under otherwise identical conditions, which demonstrates the effect of ionic charge. 0.2 M Na_2HPO_4 gave $\varepsilon_{580}/\varepsilon_{540} = 1.0$ (for 5×10^{-5} M ferriheme at pH 9). 2 M NaCl and NaBr exhibited similar visible range spectra. Comparing equal concentrations of LiBr and NaCl up to about 1 M, the former showed a much larger spectral effect. (At 5×10^{-5} M ferriheme the values for LiBr were closer to the upper curve of Fig. 2). It appears that the present three-component system conforms to salting-out relations and that both ionic strength and radii are important. 0.6 M $CaCl_2$ or $MgSO_4$, and 2 M LiBr caused rapid coagulation.

Evidence for aggreggation between ferriheme molecules at various salt concentrations has been presented by Shack and Clark[6] by measurement of sedimentation and diffusion (see also references cited therein). We have observed that 4×10^{-4} M ferriheme at pH 10.5 in the presence of 0.74 M NaCl sedimented broadly with an average of about 3 S at 20°C. Similar *s*-values were obtained with 3.6×10^{-3} M poly-DL-lysine in the presence of 4×10^{-4} M ferriheme while the pure polypeptide sedimented more slowly. On the other hand, 4×10^{-4} M

ferriheme alone at pH 11 – 12 did not produce any detectable sedimentation pattern at 50 740 rpm. The diffusion coefficient was about twice as large for ferriheme (D of the order 40×10^{-7} cm^2sec^{-1}) in the absence of NaCl as compared to ferriheme in 0.7 M NaCl. Very fast moving colored peaks ($\approx$ 45 S) were observed in the system ferriheme: poly-L-lysine (polymer 4.5×10^{-3} M; mol. wt. $\approx$ 50 000; NaCl 0.74 M; ferriheme 4×10^{-4} M; pH 9). Most of the polypeptide sedimented more slowly (1.1 S) and remained colorless indicating that it is unbound[10]. The spectrum of this system was similar to curve 3, Fig. 1. It therefore seems that the highly ionized poly-L-lysine forms high aggregates of ferriheme which also exhibit blue shifts in the visible range relative to ferriheme-salt. The number of lysine residues necessary to produce the green complex appears to be less than 4–5. Using an excess of highly ionized di- and trilysine at pH 9, precipitates were formed with ferriheme. The supernatant gave spectra intermediate between curves 1 and 3 of Fig. 1, while tetralysine showed a full green spectrum at pH 9 but not at low degrees of ionization (pH 11).

The similar spectral changes in ferriheme brought about either by interaction with (random) polybases or by high electrolyte concentrations also suggest that higher aggregates are formed in both cases. Aggregated dye systems have been investigated extensively in recent years (*e. g.* Ref. 11). For example in acridine orange binding to a polyelectrolyte and salting-out will both produce similar spectral effects[12]. In the present case the main band in the visible range is in the α-band region. However, upon aggregation, one may consider possible interactions between identical transition moments of neighboring and suitably oriented ferriheme molecules and changes in the polarizability of the medium which may cause splitting and shifts[13]. Hypochromism is not apparent in the present system, not even in the Soret band (Fig. 1) where the ratio of the oscillator strengths f of salt-free and highly aggregated ferriheme was estimated to 1.1:

$$f_1/f_2 = \int \varepsilon_1 d(1/\lambda) / \int \varepsilon_2 d(1/\lambda) \qquad (1)$$

The described spectral data combined with previous[6] and present sedimentation experiments establish the link between the visible spectrum of ferriheme and its state of aggregation. With polylysine or other polybases, aggregation is supposed to be conditioned by electrostatic interactions between the positively charged basic side groups and the porphyrin carboxylates. The ferriheme molecules may then interact optically by being stacked. The low-spin values obtained at high ratios of ε-amino groups of polylysine to ferriheme at pH 8–9 have previously been accounted for[14] by coordination of one or two ε-amino groups to the iron despite the high degree of ionization of the polypeptide. The magnetic moment has not yet been measured under the present conditions. Salting-out by electrolytes may lead to several types of micelles in which the ionized carboxyl groups point outwards so as to minimize their electrostatic interaction[15]. The present data did not permit an exact analysis of the equilibria involved. Most of the results seem to indicate that the free energy of formation $\triangle F_f$ of the reversible aggregates by electrolytes is mainly determined by screening of the repulsive potential between ferriheme carboxylates, by the entropy changes due

to differences in the structure of water, in particular around hydrophobic regions, and also by the various enthalpies of interaction:

$$\triangle F_f^0 = \triangle F_{el}^0 - T\triangle S_w^0 + \sum_i \triangle H_i^0 \quad (2)$$

Other terms are assumed to be relatively smaller. Further work is in progress to evaluate these quantities.

The green complex-type of spectrum observed in alkaline pyridine or dimethyl formamide[16] may indicate aggregation, although sedimentation experiments on the former system did not reveal any aggregated species.

According to Lemberg & Legge[17] "It is still a matter of controversy whether hematin in alkaline solutions is to be considered polymeric, dimeric or monomeric". The present data show that both salt and ferriheme concentration promote aggregation of the latter and that significant spectral changes occur also in the visible region upon aggregation. It may therefore now be possible to resolve some earlier discrepancies. The spectral data given for ferriheme at high pH or in borate buffer of pH 10 (quoted in Ref. 17, p. 173) which include a band maximum at 580 – 590 mμ apparently relate to the higher aggregates. The ferriheme spectrum obtained at pH 11 – 12 in the absence of external salt is possibly that of a ferriheme dimer[6] or oligomer. Recent experiments in the Soret band region at very high dilution[18] seem to confirm these relations.

The present data also emphasize the importance of evaluating the effect of buffer ions and other electrolytes as well as that of the ferriheme concentration whenever physical or catalytic properties of ferriheme are being investigated.

Acknowledgments. Thanks are due to Dr. A. Ehrenberg for valuable discussion and to Mrs. T. Robinson for skilful technical assistance.

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Received April 1, 1963.