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Haem-apoprotein interactions detected by resonance Raman scattering in Mb- and Hb-derivates lacking the saltbridge His146β-Asp94β

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Abstract. The dispersion of the depolarization ratio of oxidation- and spin-marker lines of sperm whale myoglobin derivatives (oxyMb, deoxyMb, ferric Mb-CN) and of ferric Hb-CN have been measured for different pH-values in the acid and alkaline region. No pH-dependence in the region above pH = 6.5 has been found. Below pH = 6.5, however, a significant pH-dependence of the oxyMb-oxidation marker line at 1,375 cm^{-1} exists. Additionally, a weak pH-dependence of the corresponding 1,355 cm^{-1} line of the deoxymyoglobin spectrum is observed. This effect can be explained assuming a titration of distal histidine, inducing a rupture of the ligand-imidazole H-bond in the case of oxymyoglobin. The pH-independent depolarization ratio disperson above pH = 6.5 in all systems investigated is explained by the lack of the haemoglobin saltbridge between His(HC3)β and Asp(FG5)β, which is essential for the cooperativity in the haemoglobin system.

Key words: Oxymyoglobin, deoxymyoglobin, metmyoglobin-cyanide, methaemoglobincyanide-resonance Raman scattering, dispersion of depolarization ratio

Introduction

The observation of the excitation profiles (EP) and depolarization ratio (DPR) of the resonance Raman scattering in haem proteins is a valuable tool in detecting distortions which lower the symmetry of the porphyrin prosthetic group from its ideal D_{4h}-symmetry. In this ideal symmetry, in which the group exhibits Raman lines with Raman tensors transforming like A_{1g}, B_{1g}, A_{2g}, or B_{2g}, the DPR's of all these lines are independent of the exciting wavelenght (Spiro and Strekas 1974).

Recently, Schweitzer-Stenner et al. (1984a, b) have reported on DPR-wavenumber-dispersion of some prominent haemoglobin Raman lines. These are caused by symmetry lowering static distortions of the haem group resulting from its asymmetric side chains and from haem apoprotein interactions. In the lower symmetry the new Raman tensors can be written as a linear combination of A_{1g}, B_{1g}, A_{2g}, and B_{2g}-tensors of the D_{4h} group and thus mix into each other (Schweitzer et al. 1982). This leads to wavelength dependent DPR.

Thus, for instance, the oxidation marker line at 1,375 cm^{-1} in oxyHb, which is due to an A_{1g}-vibration, should show a constant DPR, $\varrho = 0.125$, in ideal D_{4h}-symmetry. In reality, due to symmetry lowering distortions, a complicated minima-maxima structure is observered in the region between the α- and Soret absorption bands with a variation of the DPR between 0.15 and 0.30. Deviations of DPR from the ideal D_{4h}-values are also obtained for the lines at 1,584 cm^{-1} (A_{2g}) and 1,638 cm^{-1} (B_{1g}). Furthermore, all these lines show a striking dependence of DPR and EP on the pH-value of the Hb solution, which results from pH-dependence of the quarternary or tertiary structure of the protein. Thus, the investigation of pH-dependence of DPR and EP can be used to monitor pH-induced changes of the protein structure. Schweitzer-Stenner et al. (1984b) have interpreted their results by using fifth-order time dependent Loudon-theory, including static distortions, to fit the EP and DPR. By this procedure, they obtain parameters which are linearly related to the magnitude of symmetry-classified static distortions. In oxyHb, these distortions exhibit extrema at the pH-value of maximal proton release, due to the Bohr

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List of abbreviations: DPR: depolarization ratio; EP: excitation profile; oxyMb: oxymyoglobin; deoxyMb: deoxymyoglobin; metMb-CN: ferric myoglobincyanide; metHb-CN: ferric haemoglobincyanide effect. Schweitzer-Stenner et al. have proposed that the main cause of the structural changes of the EP and DPR in oxyHb is a pH induced rupture of the saltbridge between Asp94 (FG1) β and His146 (HC3) β. They have performed their experiments in phosphate free solutions and at Cl^- concentrations less than 0.4 M. In this case, the saltbridge can also exist in the oxygenated (R-state) of the molecules (Russu et al. 1980, 1982). This suggestion is supported by the fact that upon modification of oxyHb with BME (bis-N-maleimido methyl ether), which prevents the existence of this bridge, the pH-dependence is drastically reduced in all observed lines (Wedekind et al. 1984).

In this paper we report on measurements of EP and DPR of Raman lines in deoxy- and oxymyoglobin, metmyoglobin-CN and methaemoglobin-CN. In all these systems cooperativity of ligand binding and Bohr-effect are missing. Furthermore, none of them contain this particular saltbridge. Our experimental finding is that above pH = 6.5 no dependence of EP and DPR on pH was found in all systems studied.

Experimental

a) Material

Myoglobin from sperm whale, in lyophilized form, was obtained commercially (Miles). Crystals were grown according to the procedure described by

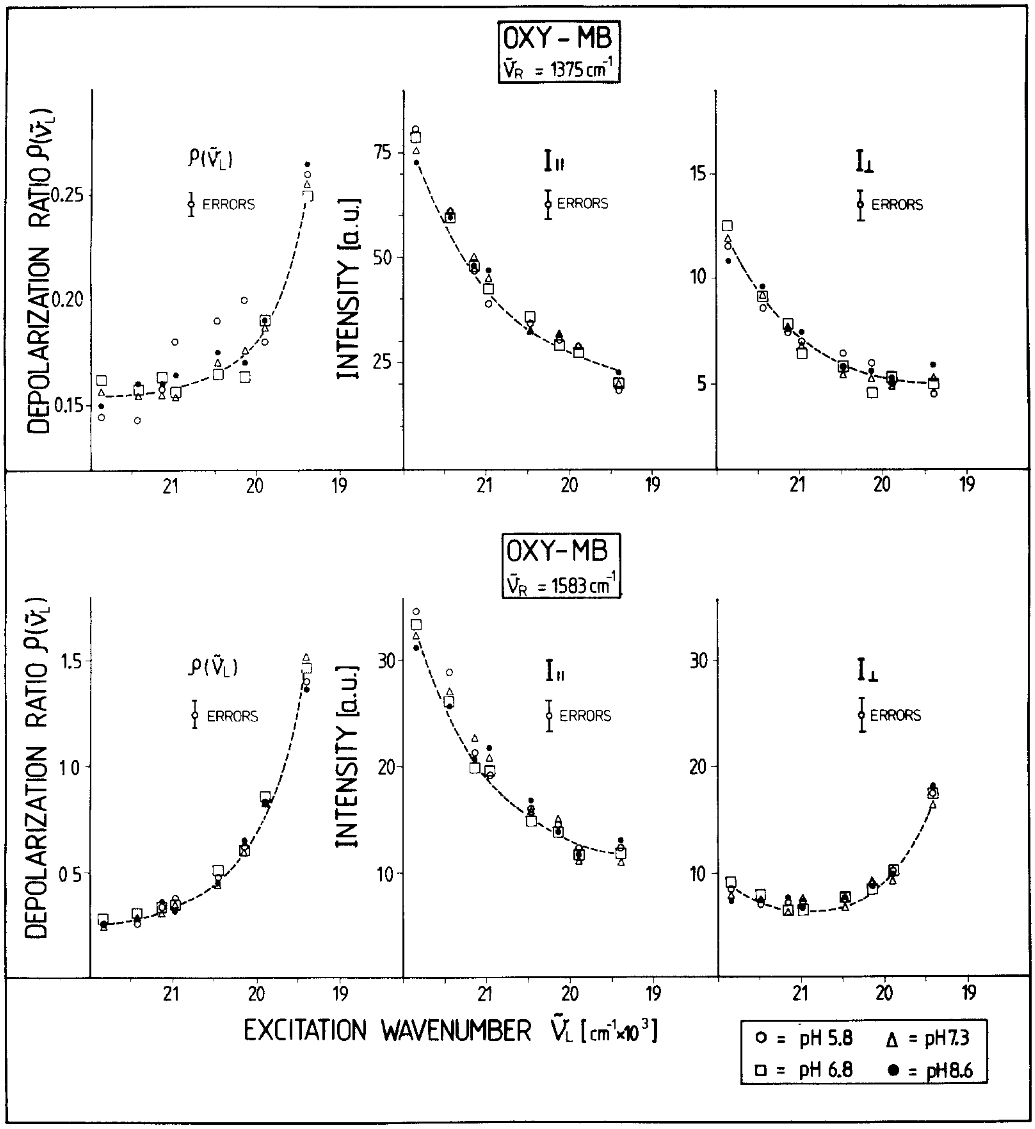

Fig. 1. DPR dispersion curves and EPs of the 1,375 cm^{-1} and the 1,583 cm^{-1} Raman lines of the oxymyoglobin spectrum for different pH-values of the solution. The dashed lines represent the averaged shape of the curves

Kendrew and Parrish (1956), Takano (1977). The crystals were washed several times in a solution of 3 M $(NH_4)_2SO_4$ and were then dissolved in bi-distilled water. To obtain salt-free solutions this solution was dialyzed against bi-distilled water.

To obtain oxyMb the solution of metMb was dialyzed against 0.01 M *tris*-buffer, pH 8.0, at 4° C for 24 h and was stored in a nitrogen atmosphere. A 1 M, oxygen free solution of $Na_2S_2O_4$ in *tris*-buffer, pH 8, was mixed with the metMb solution until a concentration of 0.1 M $Na_2S_2O_4$ was achieved. The solution of deoxyMb, thus obtained, was passed down a Sephadex G25 column of 2 cm diameter and 80 cm length. The main fraction of the effluent consists of MbO_2, which can be stored for several weeks at 4° C without significantly oxidizing to metMb.

DeoxyMb was prepared by addition of a $Na_2S_2O_4$ solution, under a nitrogen atmosphere, to a metMb solution. MetMb-CN was obtained by adding KCN to the metMb solution. The concentration of KCN was less than 0.01 M. The pH values between 5.5 and 7.0 were adjusted with 0.01 M *bis-tris* buffer and those between 7.0 and 9.0 with 0.01 M *tris* buffer.

MetHb-CN was prepared by oxidation of oxyHb with $K_3Fe(CN)_6$, followed by dialysis against bi-distilled water. The solution was run through a Sephadex G25 column and KCN was added to the main fraction.

The quality of all samples was monitored by absorption spectrometry with a Cary 17 D. The concentrations of the solutions used to obtain the Raman-spectra were about $5 \cdot 10^{-4}$ M.

b) Method

The exciting radiation was obtained from an Argon-ion laser. The laser beam, polarized perpendicularly to the scattering plane was focused, by a cylindrical lens, onto the sample, which was situated in a copper block for cooling (temperature ≈ 6° C). The Raman radiation was measured in back-scat-

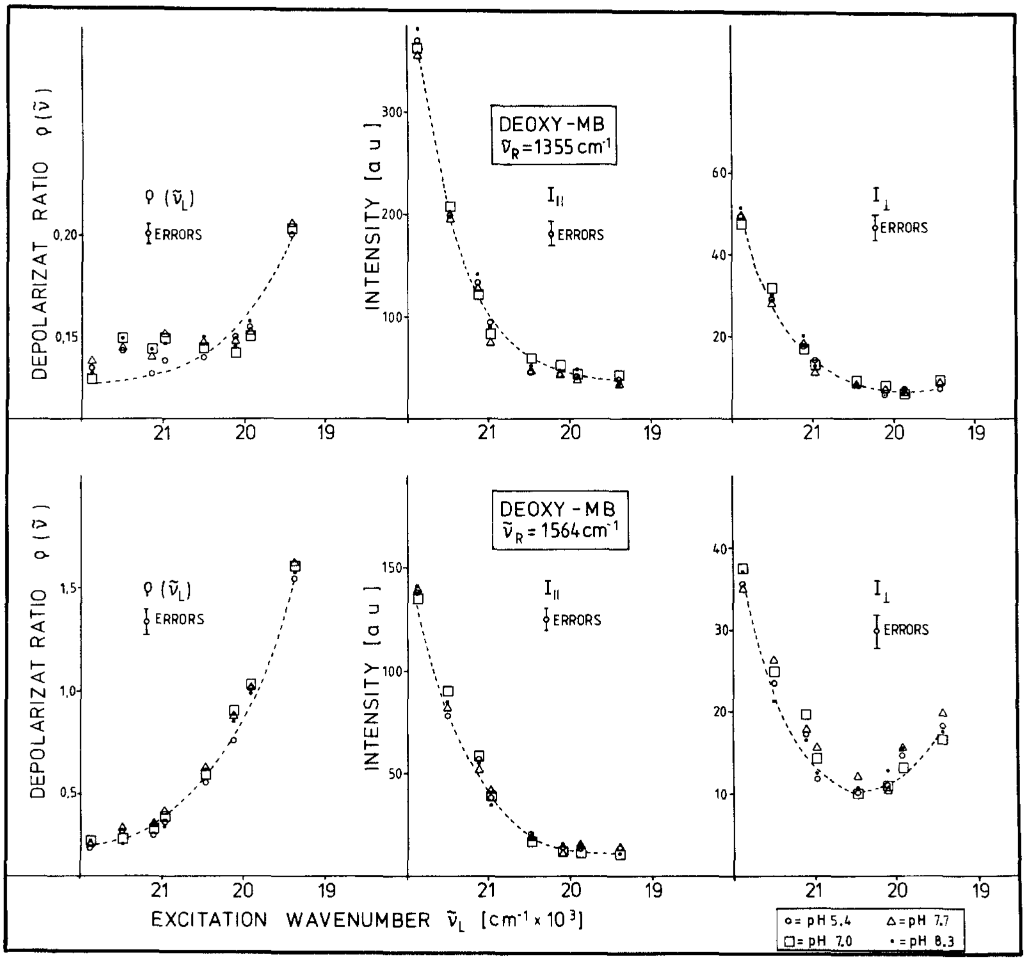

Fig. 2. DPR dispersion curves and EPs of the 1,355 cm^{-1} and the 1,566 cm^{-1} Raman lines of the deoxymyoglobin spectrum for different pH-values of the solution. The dashed lines represent the averaged shape of the curves

tering geometry. A polarizer between the sample and the entrance slit of the Czerny Turner double monochromator enables us to measure the intensity of the two components, perpendicular ($I_\perp$) and parallel ($I_\parallel$) to the E-vector of the laser beam and the DPR, $\varrho = I_\perp/I_\parallel$. To eliminate the different transmission of the spectrometer for the two components, a polarization scrambler was placed between polarizer and entrance slit.

To obtain the excitation profiles of the Raman lines we have taken into account the transmission dispersion of the polarizer and the spectrometer. The transmission of the polarizer has been measured with a Cary absorption spectrometer. The spectral response of the spectrometer system including the photomultiplier was determined by measuring the Raman-intensity of several lines of calcite and quartz for each laserline of the Ar^--laser, and correcting for the $\tilde{\nu}_R^4$-frequency dependence. This is possible since these materials have a frequency-independent Raman tensor. Since in our spectrometer we use a ruled grating, which does not show any anomalities in transmission, the spectral response of the spectrometer system can be obtained by interpolating between the measured points.

A correction for absorption of the samples is not necessary since the absorption of the solution for the concentration of maximal 10^{-3} moles/monomer is in the order of $8\,cm^{-1}$. The length of the scattering volume, imaged to the entrance slit is about 100 m, which is by a factor of 10 smaller than the penetration depth of the exciting radiation into the sample. All data given in Figs. 1–4 are calibrated to a haem-concentration of $8 \cdot 10^{-4}$ M.

The collection cone of the backscattered radiation had an halfangle of 30°. According to the calculations of Deb et al. (1984) on the error in DPR due to finite collection angles this produces a maximal error of + 6‰ from the real DPR-value for lines with $\varrho = 0.125$; for lines with $\varrho = 0.75$ the maximal error is + 1,7% and for $\varrho = 2$ the maximal error is – 6%. This error is almost exclusively from the $I_\parallel$-component of the radiation, whereas the error is the $I_\perp$-component is practically negligible. Since all these errors are in the range of our statistical errors, we have not corrected for them.

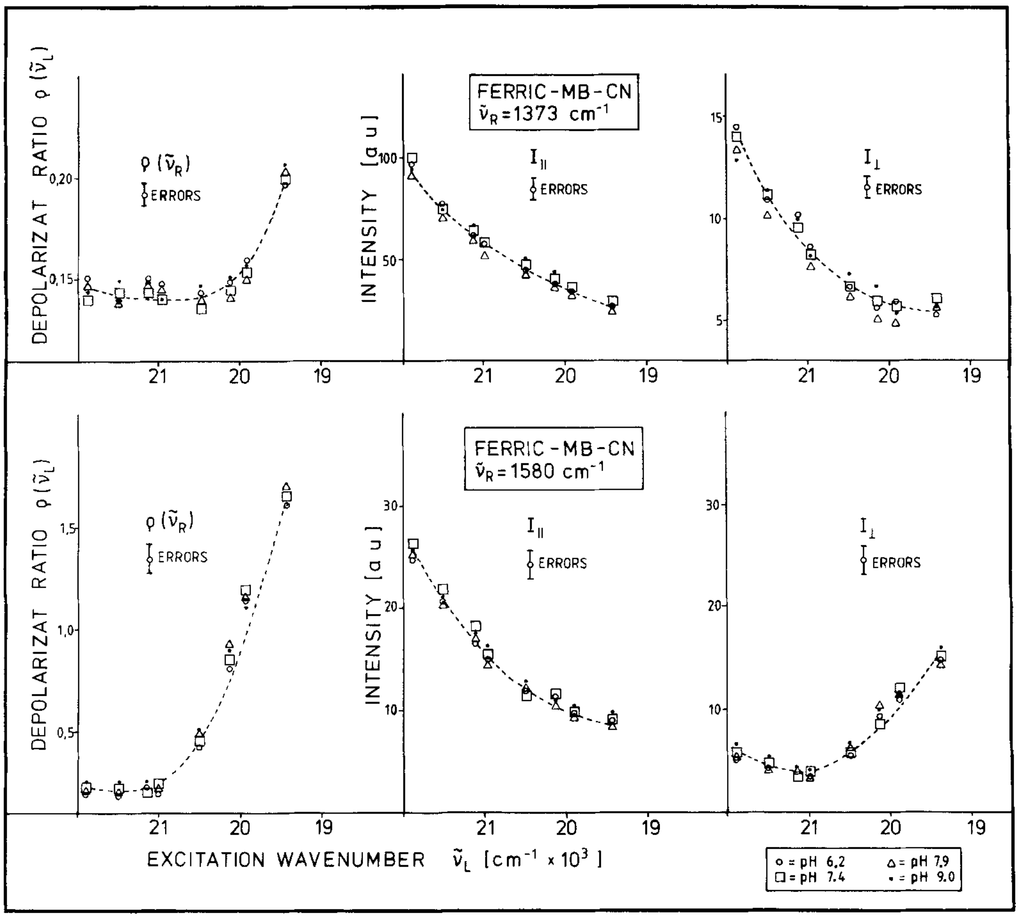

Fig. 3. DPR dispersion curves and EPs of the 1,373 cm^{-1} and the 1,580 cm^{-1} Raman lines of the ferric myoglobin cyanide spectrum for different pH-values of the solution. The dashed lines represent the averaged shape of the curves

Results

Figure 1 shows the DPR and EP of the Raman lines at 1,375 cm^{-1} (A_{1g}) and 1,580 cm^{-1} (A_{2g}) for oxyMb at various pH values. Within the accuracy of measurement no variation with pH above pH = 6 is observed. At pH = 5.9 the points deviate from those at higher pH, indicating a pH-dependence in the acid region similar to that for deoxyHb (Schweitzer-Stenner et al. 1984a). The DPR of the A_{1g}-line varies between 0.125 and 0.20, showing that symmetry lowering distortions are present. The A_{1g}-line shows resonance enhancement in the EPs towards the Soret-band. The DPR of the A_{2g}-line changes from 0.25 to 3. In ideal D_{4h}-symmetry it would have $\varrho = \infty$. The EPs again show resonance enhancement toward the Soret-band for $I_{\parallel}$, but also enhancement towards the β-band for the $I_{\perp}$-component. Figure 2 gives the results for deoxyMb. These are similar to those of Fig. 1. The DPR of both lines, however, is closer to the values of $\varrho = 0.125$ (A_{1g}) and $\varrho = \infty$ (A_{2g}) for ideal D_{4h}-symmetry, thus indicating that the porphyrin group is nearer to its ideal symmetry in deoxyMb than in oxyMb. This is reasonable since one expects constraints to be imposed upon the haemgroup by ligand binding. Again, within the error of measurement no variation with pH is observed above pH = 6. The values of ϱ at pH = 5.4 are lower in the region of 21,000 cm^{-1} for the A_{1g}-line. This might indicate a weak pH-dependence.

It should be noted here that measurements of the pH-dependence of the corresponding A_{1g}- and A_{2g} lines in oxyHb have shown that the A_{2g}-line is far less sensitive to pH-induced changes, whereas the A_{1g}-line shows a much stronger dependence on those changes (Schweitzer et al. 1982). Therefore, structural changes between the different derivates can be monitored with greater sensitivity by comparing the DPR and EPs of the A_{1g}-lines. Figure 3 shows the results for metHb-CN. The EPs show a much weaker increase towards the Soret-bands than with deoxyHb. This is due to the shift of the Soret-band, which lies at higher energy in the case of metMb-CN. Again, especially at higher energies the value of the DPR is

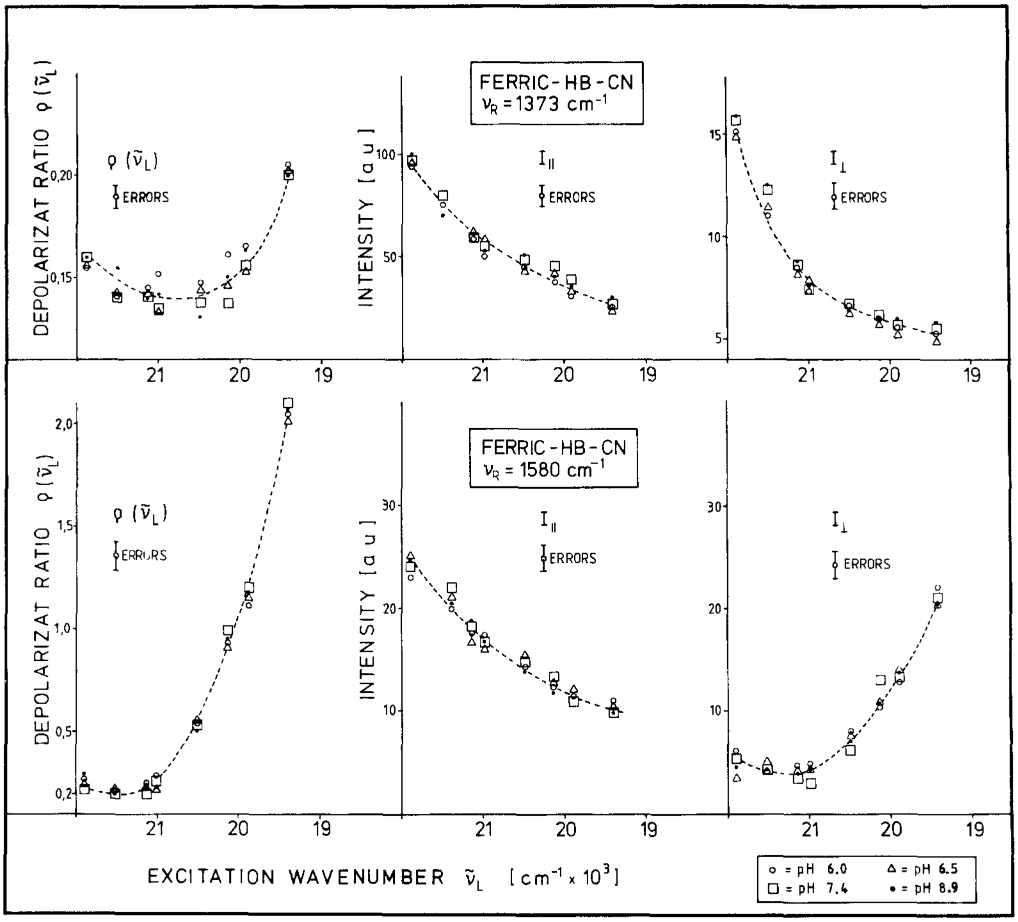

Fig. 4. DPR dispersion curves and EPs of the 1,373 cm^{-1} and the 1,580 cm^{-1} Raman lines of the ferric haemoglobin cyanide spectrum for different pH-values of the solution. The dashed lines represent the averaged shape of the curves

higher than in the deoxy derivative, indicating steric constraints in CN-binding.

Figure 4 shows the corresponding values for metHb-CN. These results are practically identical with those of metMb-CN, showing that for both proteins, CN^--binding is mainly responsible for the distortions of the haemgroup. Again, no variations of DPR and EPs with pH are observed.

Discussion

Our experiments indicate a weak pH-dependence at pH = 6.5, above this value no pH-dependence is found. We will first discuss the low pH region. La Mar et al. (1978) have shown, by proton NMR, that below pH = 6.5 sperm-whale deoxyMb exhibits a well defined pH-induced structural change. This is due to a single titrable group with a pK of 5.6. In the acid region, the haem-apoprotein contacts are looser than in the alkaline region. It was considered that the titrable group was most likely to be His(FG2) (parallel to the haem plane and over pyrrole III on the proximal side), although the distal histidine His(E7) could not be excluded. Thus, at values below pH = 6.5 the molecules are in equilibrium between two configurations. Above this value only the deprotonated conformation exists. From these findings we can understand the weak pH-dependence we have found below pH = 6.5.

A similar situation has also been found in deoxyHb, where the DPR and EP of the 1,355-cm^{-1} line show a pH-dependence below 6.5, which results from a titrable group with a pK of 5.4 (Schweitzer-Stenner et al. 1984a). In the pH range between 6.7 and 7.6 no pH-dependence was observed.

In the ligated forms, Mb-O_2 and Mb-CN, the situation is similar. Using NMR, Ohms et al. (1979) have observed protonation of the distal histidine with a pK of 5.7. In metMb-CN, by use of ^{13}C NMR, titration of either proximal or distal histidine was observed with a pK of 5.3 (Wilbur and Allerhand 1977). Although titration of other histidines more distant from the porphyrin have been found at different pK values, we can still assume that major structural changes in the haem-pocket are not induced by these groups and that the major reason for structural changes is deprotonation of the His(E7)-imidazole, which breaks the H-bond between this group and the oxygen-ligand (Shaanan 1980).

From our findings we can conclude that above pH = 6.5 no further structural changes of the haem pocket are induced. We will discuss this situation by comparison with the data obtained with oxyHb. As we have already stated, the pH-dependence of DPR in oxyHb is intimately connected with the rupture of the saltbridge between Asp94β and His146β at Cl^- concentrations below 0.4 M and at concentrations of phosphate below 0.1 M. This is supported by the fact that at higher Cl^- concentrations, where this bridge can no longer exist in oxygenated haemoglobin (Russu et al. 1980), the variations of DPR an EP with pH are drastically reduced (Wedekind et al. 1984).

The observations on BME-modified oxyHb, where the saltbridge is also missing in both the oxy- and deoxy-state (Perutz 1970a, b), and no variations of DPR and EP with are found, gives support to the importance of this bridge to the structural changes of the haem pocket (Wedekind et al. 1984).

From these facts our findings on metHb-CN become plausible. Deatherage et al. (1976) have studied the structure of horse metHb and metHb-CN by X-ray Fourier difference analysis. Although they find many drastic changes upon ligation, no difference is observed in the region of the terminal histidine, His146β. From the work of Kilmartin et al. (1980) and Heidener et al. (1976) it is known that the saltbridge is not formed in metHb, we can therefore conclude that it is also non-existent in metHb-CN. Thus, the essential trigger for pH-dependent changes of the haem environment is missing and no pH-dependent variations are observed.

The existence of this bridge is intimately connected with cooperative binding and the Bohr effect, as is shown by studies on BME-Hb, which shows neither cooperativity (Hill constant $n = 1$), nor Bohr effect (Simon et al. 1971).

From this one can understand that no cooperativity of CN^--binding is found in metHb-CN (Anusiem et al. 1968). This is in agreement with observations of Gibson et al. (1969), which show that the reaction rates for CN^--binding in the isolated α- and β-chains are identical and are equal to the binding rates of the native protein. Furthermore, the binding energies of CN^- are of comparable size, i.e., 12.0 kcal/mole in metHb-CN, (Anusiem et al. 1968) and 11.56 kcal/mole in metMb-CN (Blumenthal and Kassner 1980). We may thus conclude that binding of CN^- to metHb is similar to binding in the monomeric metMb-CN. This explains the similarity of the observed DPR and EP in both materials and their pH-independence since the saltbridge is missing in both systems.

In both oxy- and deoxyMb there also is no saltbridge and correspondingly neither cooperativity nor a remarkable Bohr effect. Parallel to this there is also no change in EP and DPR above pH = 6.5.

The importance of saltbridges in monomeric materials as an important pH-dependent trigger modulating O_2-binding affinity is also known from CTT Hb IV (Christahl and Gersonde 1982). This material, although monomeric, shows a Bohr effect by rupture of a saltbridge between the C-terminal carboxyl group (MetH22) and the imidazole in HisG2 (Parak and Kalvius 1982).

We summarize our results as follows:

1. In the acidic region below pH = 6.5 protonation of the distal histidine imidazole induces distortions in the porphyrin via changes of the haem-environment, most likely rupture of a H-bond between ligand and imidazole.

2. The absence of any structural changes at pH > 6.5, observable by changes of EP and DPR in deoxy and oxyMb, as well as in metMb-CN and metHb-CN can be explained by the fact that, in these materials, no pH-dependent trigger exists for modulating the haem-environment and thus the binding affinity of the ligand. These findings support earlier interpretations of variation of EP and DPR in oxyHb at low Cl^- and phosphate concentrations, where the rupture of the saltbridge between Asp94β and His146β is found to be the most important trigger inducing structural changes of the haem moiety above pH 6.5.

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