Vol. 86, No. 3, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 14, 1979 Pages 585-593

RELAXATION PROCESSES AND CONFORMATION OF AQUOMETHEMOGLOBIN

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Received December 11, 1978

SUMMARY. We found out that the optical extinction E of MetHbH$_2$O, pH 6 depends non-monotonously on temperature in room temperature range. The corresponding more-component equilibrium was analysed by T-jump measurements. Three groups of relaxations were observed with relaxation rates differing by more than 7 decades. Newly discovered was a relaxation in the sec-time range. The stationary temperature dependence of E is quantitatively explained by the sum of the relaxation amplitudes. Modifications of the reactive SH-groups change the rate in the ms-time range and the stationary extinction. These changes are coupled: both observables depend characteristically on the specific modification.

INTRODUCTION. In alkaline solution ($pH > 9.5$), the temperature dependence of the optical absorption of methemoglobin is determined according to ref. (1) by a two state thermal equilibrium between the high and low spin form of hydroxymethemoglobin. In the pH-region $6.5 < pH < 9.5$ it is caused by the ionization reaction of aquo- to hydroxymethemoglobin. Near pH 6.0 the situation is still unclear. Differing and contradicting arguments exist about the rôle of an aquomethemoglobin species with a low spin ground state (2-7). Therefore we analysed first the non-monotonous stationary temperature dependence of the optical extinction E of MetHb, pH 6 observed here by temperature jump techniques. Special attention was then paid to the spin-coupled ms-relaxation. The influence of dimerisation and SH-modification on the relaxation rate was investigated to find out protein regions which determine this relaxation.

Abbreviations: PMB: paramercuribenzoate; IAASL: N-(1-oxyl-2,2,6,6 tetramethyl-4 piperidinyl iodoacetamide; IAA: iodoacetamide

0006-291X/79/030585-09$01.00/0

MATERIALS AND METHODS. Oxy-hemoglobin was prepared from fresh human blood by the method of Benesch et al. (3), stripped of organic phosphates and oxidized to MetHb with $K_3Fe(CN)_6$. All solutions were used within 3 days, after preparation. For the measurements all solutions were diluted to the appropriate concentration with phosphate buffer, usually pH 6.0 with ionic strength 0.1. For modification with paramercuribenzoate, MetHb was mixed with a paramercuribenzoate solution in a ratio of one mol of reagent per mol of SH-groups β93. Modifications with iodoacetamide and N-(1-oxyl-2,6,6-tetramethyl-4-piperidinyl) iodoacetamide were carried out as described by Benesch et al. (8) and McConnell et al. (9). Measurements were done with four devices:

$\boxed{1}$. Spectral photometer acta V (Beckmann). Sensitivity to extinction changes ΔE and stability ($\Delta E/\Delta t$) were improved with external devices to ΔE=0,02/full scale and $\Delta E/\Delta t < 10^{-5}$/min. Sample temperature (thermistor YSI, type 511, 0.7 mm diameter) and extinction change ΔE are written simultaneously on a dual channel recorder (10).

$\boxed{2}$. Temperature jump apparatus after (11) (home-made), equipped with transient recorder (Biomation 8100) and signal averager (Fabritec 1072) (10).

$\boxed{3}$. Stationary version of the temperature jump apparatus to check the consistency of the results obtained with $\boxed{1}$ and $\boxed{2}$ (home-made). Mechanical light modulation, ac-amplification and phase-sensitive detection of the signal. Stability and sensitivity better than $\boxed{1}$ (10).

$\boxed{4}$. Microwave temperature jump apparatus for observation of slow relaxations (home-made). Wavelength 12 cm, 500 W magnetron, optical cuvette (2-3 ml) in a rectangular cavity.

RESULTS AND DISCUSSION. The connection of optical and magnetic properties of MetHb has been proved both in the alcaline region (spin state equilibrium) and in the region of hydrolysis (pK = 8.05). There we found linear relations between temperature and optical extinction, see fig. 1. At pH 6, however, we found, also in phosphate-free solution, a non-monotonous temperature dependence of the optical extinction (fig. 1) which is enhanced considerably by adding inositol-hexaphosphate (not shown). This requires at least three optically different species of MetHb in equilibrium. The question came up which of the reactions involved could be connected with a change in the spin state of Fe^+. Therefore temperature jump experiments were performed exhibiting the existence of relaxations with relaxation times τ in three regions:

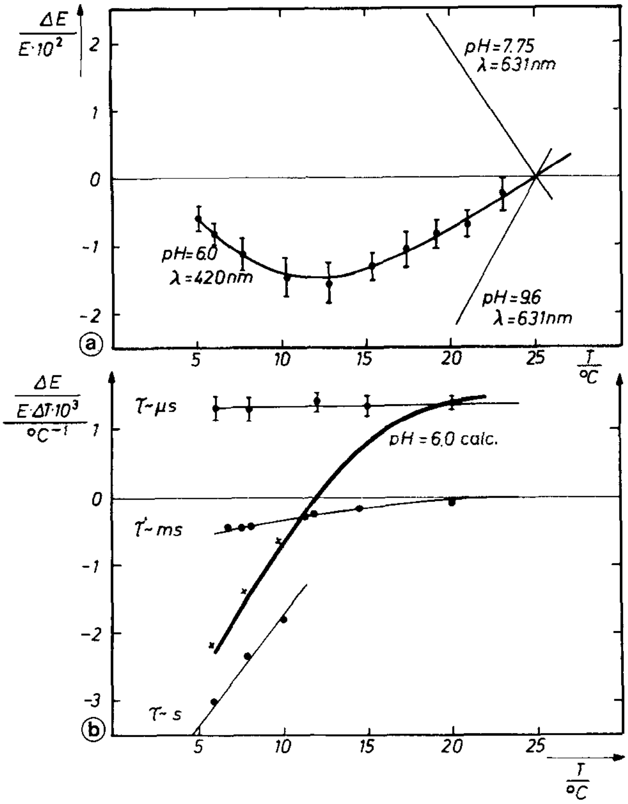

Figure 1(a). Stationarily measured temperature dependence of the optical extinction E of MetHbH$_2$O, pH 6, λ=420 nm. pH 9,6: temperature dependence due to thermal spin equilibrium; pH 7,75: temperature dependence in the hydrolysis range (pK=8,05) ; ΔE/E=(E(T)-E(25°C))/E(25°C).

1(b). Temperature coefficients of the three groups of relaxation processes described, depending on temperature, λ=420 nm, [MetHb]=1.8·10^{-5} mol·l^{-1}; (xxxx) sum of the three temperature coefficients measured; (heavy line) temperature coefficient, calculated from fig. 1a.

1. $\tau < 10 \mu s$

The relaxation amplitude ($\Delta E/E\Delta T = 1.26 \cdot 10^{-3} K^{-1}$ at λ=420 nm; 0 at λ=411 nm; $-3.3 \cdot 10^{-3} K^{-1}$ at λ=405 nm; pH 6) is temperature-independent for $4°C < T < 25°C$. The contribution from hydrolysis was calculated to be $\Delta E/E\Delta T = 1.1 \cdot 10^{-4} K^{-1}$ at λ=420 nm, which is negligibly small. The measurements in the visible region gave no clear hint to a coupling between relaxation and a change in spin of Fe^+, if one follows the criteria of ref. (1). Relaxation times could not be resolved with the measuring device. Therefore it cannot be excluded, that the temperature independence mentioned above is due to fast multistep-equilibria.

2. τ in the ms-region

Schwartz and Schimmel (12) have concluded from relaxation measurements at several wavelengths (λ=400,417,425 nm) in the Soret band that their ms-relaxation is connected with a change in spin of Fe^+. Our measurements of relaxation data in the visible (λ=560,570,630 nm) and the Soret region ($\Delta E/E\Delta T = 1.6\cdot 10^{-4} K^{-1}$ at λ=405 nm decreasing to $-4.3\cdot 10^{-4} K^{-1}$ at 420 nm) confirm this result following the criteria of George, Beetlestone, and Griffith (1). This relaxation is investigated here under conditions (aqueous solution of MetHb, pH 6, ionic strength I=0.1, $275\ K < T < 290\ K$) which are to deviate as little as possible from those where the coupling between paramagnetic susceptibility and optical absorption of MetHb has been proved (1). Another relaxation originally found by Dreyer and Ilgenfritz (13) was observed here, too, but not investigated further.

3. τ in the s-region

Newly discovered was a relaxation process with relaxation times $\tau > 10$ s. Its existence could be shown qualitatively with the apparatus $\boxed{2}$. After that it was studied in more detail with the microwave temperature jump apparatus $\boxed{4}$ designed especially for this purpose. This was done first under special conditions. At λ=420 nm (pH 6, I=0.1, stripped Hb like above, [diphosphoglycerate] < 0,015·[MetHb, tetramer]) a temperature jump from 1.2°C to 6°C caused an extinction response $\Delta E/E = -17.7\cdot 10^{-3}\cdot(1-e^{-\frac{t}{\tau}})$ with τ=33 s. Further temperature coefficients can be taken from fig. 1. No hint was found that there is a coupling to spin changes. This relaxation is still being investigated.

Fig. 1b displays, how the stationary temperature coefficient of MetHb is composed quantitatively of the measured relaxation amplitudes 1-3. The non-monotonous behaviour of E results mainly from the slow effect. In very recent investigations, Perutz et al. (14) give temperature difference spectra of MetHb, pH 7, with $\Delta T=20^{\circ}C-0^{\circ}C$. As can be seen from fig. 1, temperature difference measurements of MetHb, pH 6, with similarly large values of ΔT may give rise for mis-interpretations because the relative partition of the equilibria involved depends strongly on ΔT.

The spin-coupled ms-relaxation was analysed in more detail. Schwartz and Schimmel (12) report that the relaxation rate is drastically diminished by a factor 3 if dimerisation takes place. In comparision fig. 2 shows a rate decrement of only 30% by dimerisation. Fig. 2 displays also the relaxation rate $1/\tau=4\cdot K_{ass}[\text{dimer}]+K_{diss}$ of the tetramer-dimer equilibrium calculated with $K_D=[\text{dimer}]^2/[\text{tetramer}]=2.8\cdot 10^{-5}$ mol/l after (15) and $K_{ass}=2\cdot 10^5-5\cdot 10^5$ $mol^{-1}s^{-1}$ after (16). The rates calculated in this way lie far below those which are observed experimentally. We conclude, that the spin-coupled ms-relaxation is only to a small degree influenced by dimerisation, i.e. by the quaternary structure of the molecule. This is in contradiction to the results and conclusions in ref. (12).

The large decrease of the relaxation rate at Hb-concentrations far below 10^{-5}M which was observed in (12) and explained as dimerisation effect, needs a reinterpretation.

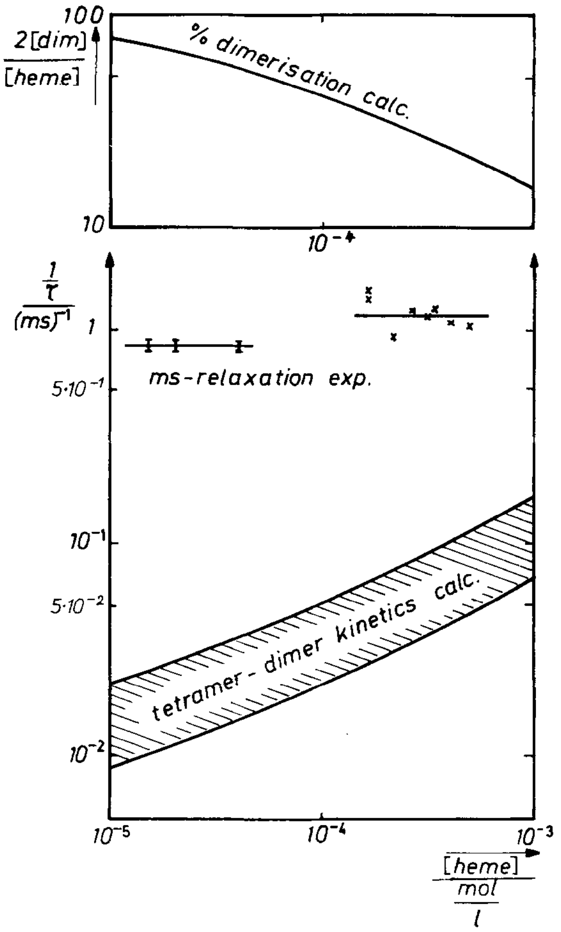

Figure 2(a). Concentration dependence of the dimerisation, calculated after (15)
2(b) top. Measured concentration dependence of the ms-relaxation. I: mean values and standard deviations from 10-15 measurements/point, λ=420 nm. x: single measurements at λ=570,630 nm.
2(b) bottom. Relaxation rate of the tetramer-dimer dissociation reaction, calculated with data from (15) and (16).

To clarify these questions further, we investigated the ms-relaxation rate for MetHbH$_2$O, pH 6, after SH-modification with PMB or IAASL. Fig. 3 shows, that modification with PMB increases the rate in the temperature range investigated. The dimerizing effect of PMB cannot alone be responsible for this, because dimerisation diminishes the rate after Fig. 3. Furthermore,

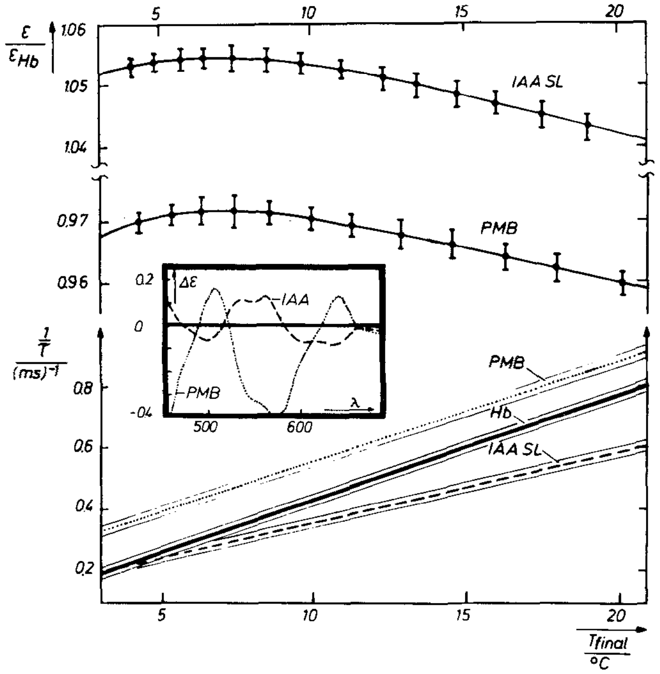

Figure 3(a). Extinction coefficient ε of modified MetHbH$_2$O, pH 6 divided by the extinction coefficient ε_{HB} of the unmodified MetHbH$_2$O, pH 6; depending on temperature, λ=420 nm.
3(b). Temperature dependence of the relaxation rate 1/τ (ms-relaxation) for unmodified and SH-modified MetHbH$_2$O, pH 6. T_{final}: temperature after temperature jump ΔT with ΔT=5.1°C. The straight lines were obtained by linear regression. The middle line is the result of the regression procedure whereas the upper and lower parallels are shifted by the standard error of the ordinate intercept.
Insert: Stationary optical difference Δε spectra between unmodified and SH-modified MetHbH$_2$O, pH 7 (after Olson (16)) (here: arbitrary units for extinction difference Δε).

IAASL has the opposite effect on the relaxation rate (Fig. 3). The situation corresponds closely to findings of Olson (16), who has shown that PMB induces high spin type spectra, whereas IAA gives rise to low spin spectra (Fig. 3, insert). A similar behaviour we found with the temperature dependence of the stationary optical extinction E of the modified protein: IAASL increases E, whereas PMB decreases E in the temperature range 10°C-25°C, see Fig. 3a. These measurements were done at

λ=420 nm, this wavelength being here a "low spin" wavelength. We conclude that the ms-relaxation rate and the optical properties mentioned of the MetHbH_2O, pH 6, are connected with each other and with the spin state of the heme iron. They are influenced by local protein structures in the neighbourhood of the proximal histidine. Because the rates observed are far above the tetramer-dimer rates and little influenced by dimerisation (Fig. 2), we propose as a simpler reaction scheme for the process, underlying the ms-relaxation (cf. (12)) $A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} B$ the states A and B differing in the spin state of the heme iron. Both states are possibly in rapid equilibrium with other states of the protein. If we identify B as the low spin state, then from susceptibility data (7) follows $K=k_1/k_{-1} < 1$. In the limit $K << 1$, the rate $1/\tau=k_1+k_{-1}$ is determined by the back-reaction k_{-1} (11). Both SH-modifications tested change K as well as $1/\tau$ in opposite directions: K is increased by IAASL and decreased by PMB; $1/\tau$ is decreased by IAASL and increased by PMB in the temperature range 5^oC-20^oC. Further work on this is in progress.

ACKNOWLEDGEMENT

We are grateful to Dr. Wenzel and G. Becker for synthesizing the spin label and measurements, to H. Lehky, S. Matussek, H. Menzel, A. Rudolf, R. Seyfarth for help in constructing the measuring devices, to H. Kasklioglou for drawing the figures, H. Grotthausmann and to K. Lackmann for typing the manuscript.

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