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Conformational transition of aquomethemoglobin: intramolecular histidine E7 binding reaction to the heme iron in the temperature range between 220 K and 295 K as seen by EPR and temperature-jump measurements

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Temperature-dependent EPR and temperature-jump measurements have been carried out, in order to examine the high-spin to low-spin transition of aquomethemogobin (pH 6.0). Relaxation rates and equilibrium constants could be determined as a function of temperature. As a reaction mechanism for the high-spin to low-spin transition, the binding of N_ε of His E7 to the heme iron had been proposed; the same mechanism had been suggested for the ms-effect, found in temperature-jump experiments on aquomethemoglobin. A comparison of the thermodynamic quantities, deduced form the measurements in this paper, gives evidence that indeed the same reaction is investigated in both cases. Our results and most of the findings of earlier studies on the spin-state transitions of aquomethemoglobin, using susceptibility, optical, or EPR measurements, can be explained by the transition of methemoglobin with H_2O as ligand (with high-spin state at all temperatures) and methemoglobin with ligand N_ε of His E7 (with a low-spin ground state). Thermal fluctuations of large amplitude have to be postulated for the reaction to take place, so this reaction may be understood as a probe for the study of protein dynamics.

Introduction

The equilibrium between high-spin and low-spin states of aquomethemoglobin (pH 6.0) was the subject of various investigations. After thinking of aquomethemoglobin as a pure high-spin state [1,2], EPR [3,5], susceptibility [5,6], and optical [5,6] measurements showed that a low-spin state exists at low temperatures. At room temperature, very different amounts of molecules in the low-spin state were found – ranging from about 0 to 22% [5–9]. However, there seems to be no doubt that the equilibrium is shifted to higher spin with rising temperature [6,8,10]. The temperature-dependent susceptibility measurements showed a complicated behaviour on freezing of the sample. Particularly, a dependence on the freezing speed was found, which could be confirmed by optical measurements [6]. The same was found in recent EPR measurements of methemoglobin (pH 7.0) [11]. Unfortunately, there are no further temperature-dependent measurements that show the spin equilibrium. One reason is that EPR measurements yield well-resolved spectra only at temperatures below 100 K.

As a reaction scheme for the high-spin to low-spin transition (at pH 6), it was suggested that the ligand H_2O is replaced by N_ε of the distal Histidin (His E7) [5,8,12]. This reaction was first found for the methemoglobin Norfolk, which has an amino acid close to the heme region of the α-subunits changed [13]. But EPR [12] and optical [8] measurements gave rise to the suggestion that this happens in human methemoglobin A too.

On the other hand, T-Jump measurements were carried out on methemoglobin (pH 6.0) at temperatures above 0 °C showing different internal reactions of the heme. Of one of these it was suggested that it is accompanied by a change in the spin state [14], this could be confirmed later [15]. Several proofs could be found that showed that this reaction observed by T-jump is also

Abbreviations: T-Jump, temperature jump; metHbH_2O, aquomethemoglobin; metHbHis, methemoglobin with N_ε of His E7 bound to the heme-iron; metMbH_2O, aquometmyoglobin.

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the binding of His E7 to the heme-iron [15,16]. For the sake of simplicity, this reaction shall be written as:

$$\underset{\text{(high-spin)}}{\text{metHbH}_2\text{O}} \rightleftharpoons \underset{\text{(low-spin)}}{\text{metHbHis}} \quad (1)$$

(assuming that the mechanism suggested is the right one).

Temperature dependent T-jump measurements provide information about the rate constants of the reaction 1, the equilibrium constant, and thus about the quantities of entropy and enthalpy of the reaction [16]. In this paper, T-jump measurements of horse methemoglobin in the temperature range 278–296 K are presented. Measurements yield an equilibrium that is extremely on the high-spin side for temperatures above 0 °C. Following the ideas of Dreyer and Ilgenfritz [15] who examined human methemoglobin by T-jump measurements, it should be possible to reach an equilibrium constant of $K = 1$ at about 230 K. When extrapolating the relaxation rates to this temperature range, values in the min-time scale are obtained.

It is difficult to investigate this reaction by T-jump and optical means in the temperature range below 0 °C, so we investigated the reaction by EPR measurements, namely to determine equilibrium constants and relaxation rates below 0 °C. If the reaction investigated by T-jump measurements is the same as the one seen in EPR, optical, or susceptibility measurements, the same results should be obtained as are expected for the T-jump measurements. Thermal fluctuations of large amplitude are necessary for binding reaction 1 to take place [16]. So this internal reaction can be taken as a probe for large-scale protein fluctuations, and the EPR measurements below 0 °C may provide information about protein conformational transitions in frozen solution at low temperatures.

Methods

Sample preparation

Oxyhemoglobin was extracted from fresh human or horse hemoglobin by the method of Benesch et al. [17], freed from organic phosphates and Cl^-, and then converted to methemoglobin by addition of a 2-fold amount of $K_3Fe(CN)_6$. For the measurements, the sample was kept in 0.1 M phosphate buffer (pH 6.0). For T-jump measurements, samples of 0.3–0.5 mM (heme concentrations) were used, whereas EPR measurements were carried out on a 4 mM solution to achieve high signal-to-noise ratios.

T-jump measurements

T-jump measurements were carried out on a home-made apparatus, built according to the scheme of Eigen and de Mayer [18], equipped with a Nicolet 1072 transient recorder and a CBM 8296 computer system. Transients of the absorbance change at $\lambda = 633$ nm following a T-jump of $\Delta T = 3.2$ K have been analysed in the temperature range between 273 and 295 K.

The reaction given in (1) can be written in a short form as:

$$A \rightleftharpoons B \quad (1')$$

The relaxation time τ of the reaction can be determined from the time-course of the absorption that follows the T-jump (of amplitude ΔT):

$$E(t) = E_0 + \Delta E(1 - e^{-t/\tau}) \quad (2)$$

The relation between relaxation time and rate coefficients is given by:

$$\tau^{-1} = k_{on} + k_{off} \quad (3)$$

The equilibrium constant K is defined as:

$$K \equiv [B]/[A] = k_{on}/k_{off} \quad (4)$$

A combination of the Eqns. 3 and 4 yields the reaction rate k_{off}:

$$k_{off} = \tau^{-1}/(K+1) \quad (5)$$

K can be determined from the T-jump data by:

$$K(T) = \frac{(1-V)Y(T)}{2X(T)T^2} + \sqrt{\left\{\frac{(1-V)Y(T)}{2X(T)T^2}\right\}^2 + V} \quad (6)$$

where ε_A, ε_B are the molar absorption coefficients of the pure states A and B. V, X and Y are defined by:

$$V = \frac{\varepsilon_A}{\varepsilon_B} \quad X = \frac{\Delta E}{E\Delta T} \quad Y = \frac{d(\ln|XT^2|)}{d(1/T)} \quad (7)$$

V was experimentally determined by comparison of the high-spin form of methemoglobin (pH 6.0) and the low-spin form of methemoglobin (pH 9.6) as $V = 0.65$.

ΔH^0 and ΔS^0 can now be calculated from the plot ln K vs. T^{-1}:

$$\ln K = -\Delta H^0/RT + \Delta S^0/R \quad (8)$$

Activation quantities were calculated according to the relation of Eyring [19]:

$$k_{off}(T) = k_B T h^{-1} e^{-(\Delta H^{\#} - T\Delta S^{\#})/RT} \quad (9)$$

EPR measurements

For the EPR measurements, a home made X-Band spectrometer, equipped with an AEG H_{103}-cavity and a

modified Oxford variable temperature accessory, was used. The modulation frequency of the magnetic field was 52 kHz, the peak-to-peak amplitude of modulation 6.5 G. The microwave power used was 11 mW, corresponding to a microwave field of 0.17 G in the cavity.

The main problem is that the broad lines of the heme can be well resolved only in the temperature range below 100 K. To get exact information about higher temperatures, one possibility is to cool the sample rapidly, strictly speaking, rapidly compared to the relaxation time. After cooling, all measurements are performed at the same temperature so that the high-spin fractions of different temperatures can be compared. Using this method, it is also possible to determine the relaxation times of certain temperatures: A sample of room temperature was put into liquid nitrogen and quickly inserted into the primary cooled cavity cryostat, reaching a maximal temperature of about 130 K. After a first measurement at 90 K, it was warmed up to a temperature T_t for a time Δt_t, and then cooled again to a temperature of 90 K for measuring. This procedure – warming up for tempering and cooling for measuring – was repeated a few times. (A temperature of 90 K instead of 77 K was chosen, because this temperature could be stabilized more precisely.) For the sake of simplicity, this is called 'method I' in the rest of the paper. This method can only be applied for low temperatures, because the relaxation times must be long compared to the time t_c required to reach the designed temperature.

To check these restrictions, experiments were first carried out to get a rough estimation of the relaxation times and the freezing speed. The following observations were made: After 16 h at a temperature of 216 K no change in the spectrum was visible, at 228 K a significant change was detected after 17.5 h, and at 239 K a relaxation time of about 1 h was found, whereas at 246 K the equilibrium was reached in less than 1 h. This result means that, at first, a reaction can be seen with relaxation times in the order of magnitude, as they would be expected according to earlier T-jump measurements [15,16]. Secondly, the reaction observed is completely suppressed, if the sample is kept at temperatures below about 215 K. This is especially important for the insertion of the sample (see above). Thirdly, in the temperature range between 228 K and about 240 K, method I is adequate to determine relaxation times. For the time required to cool the sample from temperature T_t to temperatures below 215 K, where the reaction is suppressed, was about 0.5 min; and it did not take more than 4 or 5 min before the temperature was between 215 K and $T_t - 2$ K when the designed temperature was approached. So the condition $t_c \ll \tau$ was fulfilled in the temperature range mentioned. Besides, the temperature could be stabilized for days with a fluctuation of less than 1 K.

For temperatures between 240 K and 250 K, the relaxation times are too short to be explored, but the equilibrium amplitude of the high-spin peak could still be measured, since the cooling from T_t to 215 K was still fast enough. Finally, for temperatures above 250 K, even this is not possible; the cooling must be executed more rapidly. So the following method – method II – was applied: The sample was kept in the vapour of liquid nitrogen on a temperature T_s (for a time Δt) and then put into the liquid nitrogen. The equilibrium state of temperature T_s is frozen if time Δt is long compared to the relaxation time τ, and if time t_c, necessary to cool the sample, is short compared to τ. To fulfill the first condition – $\Delta t \gg \tau$ – the sample was equilibrated for a few minutes if the starting temperatures were above 0 °C, and for a longer time (13 min–2 h) for lower temperatures T_s (265–248 K).

Time t_t has been experimentally determined by means of a thermo-element inside the sample. We have got a cooling speed of 12.3 K/s in the crucial temperature range between 253 and 268 K, when the sample is put into liquid nitrogen. To get information about temperatures above 250 K, this second method was used. For a mediate temperature of 248 K both methods were employed to check, whether they both provide the same results.

On the whole, the methods were chosen in a way that the first condition, $\Delta t \gg \tau$, is fulfilled in any case, whereas the condition $t_c \ll \tau$ was fulfilled for temperatures below about 258 K. For higher temperatures, the reaction is in equilibrium in the beginning of the cooling process. When the temperature decreases further, the relaxation is to slow to follow the freezing process. So an intermediate temperature of 261 K is frozen up, as could later be determined by means of a computer simulation. But even at this temperature, the equilibrium is strongly on the high-spin side [15].

Results

T-jump measurements

The reaction rates k_{off} are calculated from the measured relaxation times using Eqn. 5. The data are summarized in Fig. 3. Equilibrium constants, K, were calculated due to Eqn. 6. The values of K are small compared to unity in the temperature range 279 K $\leq T_s \leq$ 291 K as received before [15,16]. The data are summarized in Fig. 4

The thermodynamic parameters ΔH^0, ΔS^0, $\Delta H^{\#}$ and $\Delta S^{\#}$ could be determined from the temperature dependences of K and τ, using Eqns. 8 and 9. The results are listed in Table I. These quantities are all somewhat larger than obtained in an earlier work [16]. This is caused by an improvement of the apparatus, and not by differences between the two hemoglobin species; for T-jump measurements provided very similar results

TABLE I

Activation energies and entropies and standard differences in entropy and enthalpy

The data are obtained by T-jump measurements of horse methemoglobin in a temperature range between 295 and 275 K, and EPR measurements in the temperature range between 295 and 235 K in 0.1 mM phosphate buffer (pH 6.0). The heme concentration used was 0.3–0.5 mM for the T-jump and 4.0 mM for the EPR measurements.

Sample	Method	ΔH^0 ($kJ \cdot mol^{-1}$)	ΔS^0 ($J \cdot mol^{-1} \cdot K^{-1}$)	$\Delta H^{\#}$ ($kJ \cdot mol^{-1}$)	$\Delta S^{\#}$ ($J \cdot mol^{-1} \cdot K^{-1}$)
Horse metHb	T-jump	-65.6 ± 3.8	-274 ± 14	85.5 ± 0.3	105 ± 1
Horse metHb	EPR	-82.1 ± 10	-333 ± 41		
Horse metHb	T-jump, EPR	-79.9 ± 2.2	-324 ± 8	176 ± 3	424 ± 7

for human and horse methemoglobin (unpublished data).

EPR measurements

The amplitude, A_e, of the high-spin peak ($g = 6$) was independent of the starting temperature, T_s, in the temperature range 265 K $\leq T_s \leq$ 296 K. (The subscript e denotes amplitudes that are measured when the system was in equilibrium before the rapid cooling.) Starting at temperatures below 260 K, a decrease in the equilibrium-amplitude $A_e(T)$ of the high-spin peak was detected. This decrease in $A_e(T^{-1})$ is shown in Fig. 1. The equilibrium is shifted from the high-spin to the low-spin state between 255 and 240 K.

The two measurements at 248 K (1000 $K/T = 4.03$) yielded very similar values of A_e, showing that both equilibrating methods (I and II) provide consistent results. Thus, the results of both methods could be brought up for the analysis. When the sample was warmed up to room temperature and then again rapidly cooled in liquid nitrogen, the original spectrum was measured again. So it was verified that the reduction in peak amplitude is really caused by a high-spin to low-spin transition and not by any deleterious effect.

Fig. 2 shows the decrease in amplitude A of the high-spin peak with time at a temperature of 235.5 K (1000$K/T = 4.25$), after the sample was rapidly cooled from room temperature. Clearly, two relaxation processes can be seen. Relaxation times have been determined, supposing an exponential decrease in the amplitude A for both relaxations and – as an approximation – identical amplitudes:

$$A(\Delta t_t)/A(0) = \tfrac{1}{2}(1 - a_e)\, e^{-\Delta t_t/\tau_1} + \tfrac{1}{2}(1 - a_e)\, e^{-\Delta t_t/\tau_2} + a_e \qquad (10)$$

The relaxation times, obtained by fitting the times τ_1 and τ_2 to the decrease in peak-amplitude, are listed in

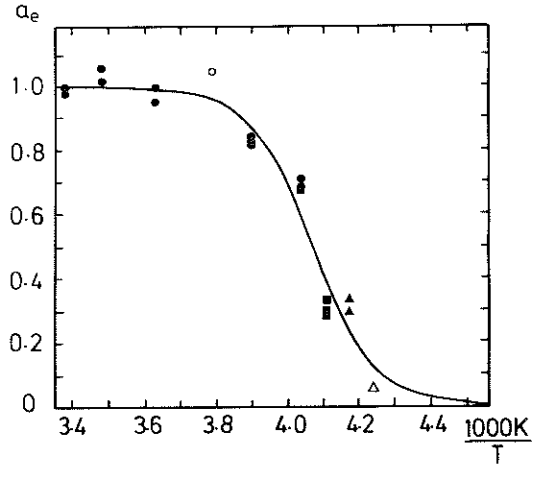

Fig. 1. High-spin to low-spin transition in aquomethemoglobin, measured by EPR. Decrease in the relative (equilibrium) high-spin amplitude $a_e(T) = A_e(T)/A_0$ of 4.0 mM horse methemoglobin (pH 6.0) in 0.1 mM phosphate buffer. A_0 is the (temperature-independent) value of $A_e(T)$ in the temperature range 265 K $\leq T \leq$ 297 K. The high-temperature data are obtained by putting the sample into liquid nitrogen after equilibrating at T_s (●, ○); in the low-temperature range, the sample is tempered in the cavity at temperature T_t (■). For the lowest temperatures, the fitted values of $A_e(T)$ are used (▲, △), because the equilibrium has not been reached within the time of measurement. Open symbols (○, △) design measurements of poorer accuracy. The line shows the fitting of ΔH^0 and ΔS^0 to the decrease in high-spin amplitude according to Eqn. 11.

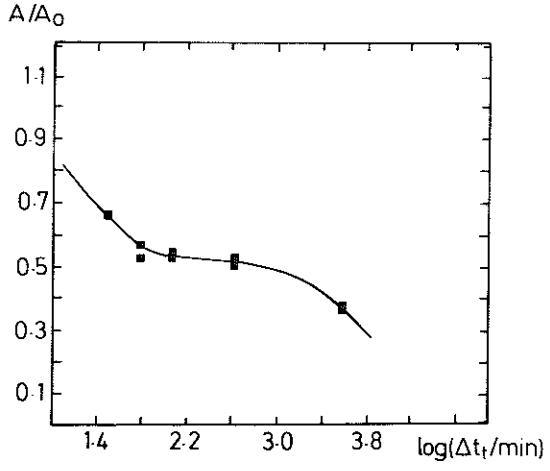

Fig. 2. Relaxation process of aquomethemoglobin, detected by EPR. Decrease of the amplitudes of the high-spin peak of 4.0 mM horse methemoglobin (pH 6.0) with time in 0.1 mM phosphate buffer. The sample was rapidly frozen in liquid nitrogen from room temperature, then tempered at 235.5 K for the time Δt_t. EPR measurements were then carried out at 90 K. The line is the fitting of Eqn. 10 to the data points.

TABLE II

Relaxation times of the reaction investigated, found by fitting the times τ_1 and τ_2 from Eqn. 10 to the decrease in peak amplitude observed in EPR measurements

The number of measurement values are given in parentheses.

T/K	τ_1/h	τ_2/h	
231	2.3 ±0.5		(4)
235.5	0.38±0.03	146 ±15	(10)
239.5		6.3± 0.7	(8)

Table II. Apart from the relaxation times, the equilibrium-value a_e of the high-spin amplitude is received. (For a precise definition of a_e, see text of Fig. 1.) The reaction rates k_{off} were determined using Eqn. 5, using values of K determined from the approximated quantities $\Delta H^0 = -90.4$ kJ·mol^{-1} and $\Delta S^0 = -368$ J·mol^{-1}·K^{-1}, see below. In Fig. 3 these results are shown together with the data of the T-jump measurements. The measurement values of both methods harmonize, though the reaction slows down more rapidly in the low temperature range than it does in the high temperature range. As an approximation (for the simulation of the cooling process), activation quantities are calculated due to Eqn. 9. Taking into account the data of both physical methods, we got:

$$\Delta H^{\#} = 176 \pm 3 \text{ kJ·mol}^{-1} \quad \Delta S^{\#} = 424 \pm 7 \text{ J·mol}^{-1}\text{·K}^{-1}$$

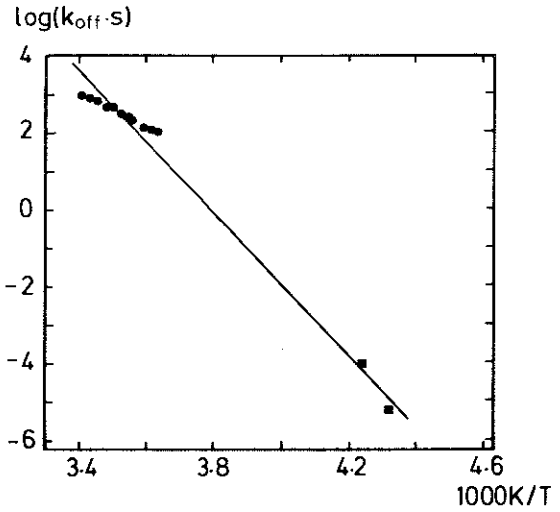

Fig. 3. Reaction rate k_{off} of the investigated reaction metHbH$_2$O ⇌ metHbHis. The data are obtained by T-jump measurements (●) and EPR measurements (■) of horse hemoglobin in 0.1 mM phosphate buffer (pH 6.0). The heme concentration used was 0.3–0.5 mM for the T-jump and 4.0 mM for the EPR measurements. The slope of the line connecting the two groups of data points is less than the slope of the regression of the T-jump data alone.

Equilibrium constants, determined from EPR measurements

To determine equilibrium constants from the EPR spectra, it is sensible to use the high-spin peak amplitude only, as the amplitude of the low-spin spectrum is difficult to determine. But the question is whether a pure high-spin state is frozen up when the sample is put into liquid nitrogen. So the following procedure was used to determine the equilibrium constants:

As a first approximation it was supposed that the maximum peak amplitude, A_0, is that of a pure high-spin state. Then the determination of the standard differences of enthalpy and entropy, ΔH^0 and ΔS^0, from the curve a_e (T^{-1}) is possible:

$$K = [\text{metHbHis}]/[\text{metHbH}_2\text{O}] = [\text{low}]/[\text{high}]$$

$$= (1 - a_e)/a_e$$

$$\Rightarrow a_e(T) = 1/(K+1) = \left(e^{-\Delta H^0/RT + \Delta S^0/R} + 1\right)^{-1} \qquad (11)$$

Here it is assumed that metHbHis is a pure low-spin state (in the temperature range 230 K $\leq T \leq$ 260 K). This assumption could be confirmed by EPR measurements of methemoglobin with imidazol as ligand. Here, a high-spin peak was not detectable. The supposition that metHbH$_2$O is a pure high-spin state can just as well be omitted: if a certain fraction of metHbH$_2$O is in the low-spin state, this leads to a low-spin spectrum to which no attention is paid, since only high-spin peaks were used for calculation. Fitting of ΔH^0 and ΔS^0 to the measured values of $a_e(T^{-1})$ gave:

$$\Delta H^0 = -90.5 \pm 11.8 \text{ kJ·mol}^{-1} \quad \Delta S^0 = -368 \pm 48 \text{ J·mol}^{-1}\text{·K}^{-1}$$

(cf. Fig. 1).

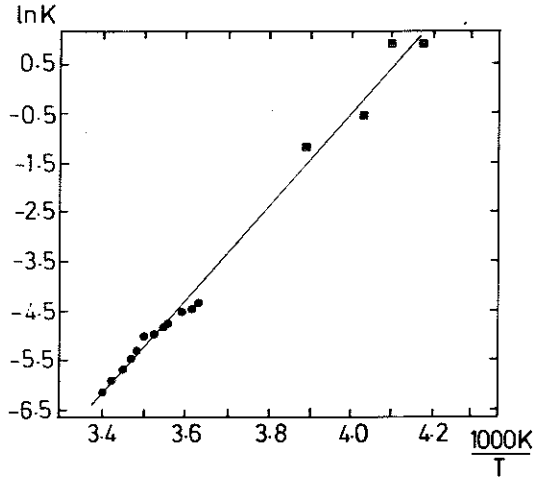

Fig. 4. Equilibrium constant of the investigated reaction. The equilibrium constant K = [metHbHis]/[metHbH$_2$O] of horse hemoglobin in 0.1 mM phosphate buffer (pH 6.0) as obtained by T-jump measurements (●), 0.3–0.5 mM heme concentration, and EPR measurements (■), 4.0 mM heme concentration. The data points obtained by the two different methods are well fitted by a single regression line.

These values permit the calculation of the temperature dependence of $a_e(T)$. The temperature-dependent relaxation times can be determined from the activation quantities $\Delta H^{\#}$ and $\Delta S^{\#}$. As the speed of the cooling process had also been measured (see Methods), it was possible to simulate the relaxation during the freezing process. For this, the temperature range from 300 to 200 K was divided into equidistant intervals for which mean values of the relaxation time $\tau(T)$ and the equilibrium amplitude $A_e(T)$ could be calculated. In this way the decrease in high-spin amplitude $A(t)$ could be simulated during the freezing process, giving a decrease of 6.5%. This corresponds to the equilibrium at $T = 261$ K, which was frozen up. This has already been mentioned in Methods. Now a correction of the relative amplitudes was possible, so that the equilibrium constants could be calculated. In Fig. 4, these constants are shown together with the data of the T-jump measurements. Here the results are very well matched. This can also be seen from the thermodynamic quantities, calculated from T-jump and EPR measurements (cf. Table I). (ΔH^0 and ΔS^0 in Table I are of course calculated from the corrected data of $A_e(T)$.)

Discussion

Comparison of T-jump and EPR measurements

The results of the two methods can be compared in spite of different experimental conditions. It has been shown that the relaxation under investigation does not depend on the methemoglobin concentration, c, in the range 10^{-5} M $< c < 10^{-3}$ M [20], so the use of a 10-fold heme concentration in the EPR experiments compared to the T-jump samples is justified in order to increase the signal-to-noise ratio. The effects of changes in pH and local salt concentrations, which might occur during freezing, had been measured. The increase in salt concentration from 0.1 to 1 M (Na_2HPO_4/Na_2PO_4, or Na_2SO_4 in phosphate buffer, or $(NH_4)_2SO_4$ in phosphate buffer) in liquid solution leads to an increase in ΔH^0 and ΔS^0 of less than 10%. The relaxation amplitude has its maximum value in the pH range around pH 6. The relaxation rates and therefore the activation parameters do not significantly depend on the salt concentration and pH in the pH range pH 5.5–7.3. So influences of possible changes in pH and local salt concentrations in the frozen solution should be small.

The equilibrium constants measured by EPR in frozen solution in the temperature range 260–236 K and those calculated from the T-jump transients in liquid solution (between 291 and 276 K) are well fitted by a single regression line (cf. Fig. 4). The data of both physical methods put together give similar values of ΔH^0 and ΔS^0 as the T-jump or EPR measurements alone (cf. Table I). So we conclude that phase transitions of water as well as changes in environmental conditions (for example pH) in the frozen solution do not grossly alter the observed reaction, though the influence of the surrounding medium cannot completely be neglected, as the following comparison of the relaxation rates shows.

The relaxation times found in the frozen state by EPR measurements are slower than expected from the T-jump measurements in liquid solution. This is not surprising, because a very simple reaction model has been used. However, complicated models had to be proposed to explain the temperature dependence of reaction rates in proteins [21]. Furthermore, the viscosity of the surrounding medium, which changes drastically at the freezing point of the solution, was neglected. It was found that protein reactions depend on the viscosity of the medium, especially if movements of the surface are involved – for heme proteins see for instance Refs. 22–24. So the deviation of the reaction rates from a behaviour according to the theory of Eyring could have been expected; it does not show that two different reactions are observed by the two physical methods. (It must be noted that great differences in $\Delta S^{\#}$ (cf. Table I) come about, because $\Delta S^{\#}$ is determined from:

$$\ln k_{off}(T^{-1} = 0) = \ln(h/k_B T) - \Delta S^{\#}.)$$

After comparison of K- and τ-values, it seems very probable that we are dealing with the same reaction in T-jump as well as in EPR measurements. Furthermore, another reason can be adduced. In our EPR measurements, two clearly different relaxation times could be observed. The same was found in recent T-jump measurements (of methemoglobin); (T-jump) measurements of isolated subunits gave a shorter relaxation time for the α-subunits (unpublished data). Here the interpretation that α- and β-subunits have different relaxation times is obvious. This is in contradiction to the general conclusion that no inequivalence between α- and β-chains is seen in high-spin ferric hemoglobin [25].

Comparison of EPR and susceptibility measurements

We would like to show now that the experimental results of the studies, cited in the Introduction, can be explained by the interpretation of a (slow) transition between two conformers – only one effect must be explained in a different way (see below).

The two conformers are called metHbH_2O and metHbHis in this paper, in order to specify the ligand of the heme iron, but they correspond to the conformers, called metHbH_2O^I and metHbH_2O^{II} in earlier studies [5,6,12]. Especially the results of the susceptibility measurements of horse aquomethemoglobin by Iizuka and Kotani [6] are of interest for our interpretation, because direct temperature-dependent measurements were carried out in this investigation. Many of these results correspond to ours. (Compare Fig. 5a of

Ref. 6 for the following explanations.) According to the cooling speed, different states at low temperatures were found. Reproducible values of the magnetic susceptibility were obtained, whilst the sample was kept below 200 K. In this temperature range, the reaction is completely frozen – relaxation times are in the time scale of days or longer. Thus, only spin transitions without a change of the ligand were possible; and these transitions took place, as can be seen from the decrease of the magnetic susceptibility with falling temperature. When the sample was warmed up to the transition region (222–250 K), reproducible results were not achieved any more. This is because transitions took place between the two conformers, with relaxation times comparable to the time required for measurements. When the cooling speed was high, a great amount of molecules were in the metHbH$_2$O and therefore in the high-spin state. When the sample was warmed up to the transition region, the transitions began to take place, as the equilibrium is on the metHbHis (or low-spin) side in the transition region. In the same way it can be understood that the spectra obtained depended on the cooling speed, because different amounts of metHbH$_2$O were frozen depending on the time to pass the transition region during the freezing process. When the temperature was raised further, an increase in the high-spin fraction was measured for all samples – independent of the freezing speed. Here the reaction is in equilibrium the whole time, and the equilibrium is shifted from the metHbHis state to the metHbH$_2$O state. The only difference between their results and ours is that they have found a sharp transition at 0 °C, while we have found a transition at temperatures below the freezing point – in accordance with earlier T-jump measurements [15]. Our interpretation cannot explain a sharp transition at the freezing point of water, and we therefore agree with the explanation given by Iizuka and Kotani that the freezing water is directly responsible for their results.

In our opinion, the reason for the different results might be the very high hemoglobin concentration of 6–12 mM (heme concentration) in the susceptibility measurements; for we found crystallization effects above 4 mM. Perhaps the crystallization is favoured by the freezing of the sample. Asakura et al. [26] have discussed the problems arising from the freezing process, which may lead to high salt or pH gradients, magnetically concentrated samples, or solvent-solute segregation. In our opinion, these difficulties grow with increasing protein concentrations. Here it must be noted that the His-binding reaction is considerably altered in crystals [27].

Spin states of the conformers

As metHbHis is in a low-spin state at low temperatures and, on the other hand, a mixture of metHbHis and metHbH$_2$O yields an intermediate spin state for $T \rightarrow 0$ K, we have to conclude that metHbH$_2$O has a high-spin ground state, provided that the ground state is not a mixed state. With rising temperature, the fraction of molecules in the high-spin state increases, independent of the ratio between metHbHis and metHbH$_2$O [6]. So it is reasonable to assume that metHbH$_2$O is in a high-spin state also at high temperatures, whereas metHbHis turns over to a high-spin or a mixed spin state with increasing temperature. We have therefore come to the same model for the spin-states that had already been suggested by Iizuka and Kotani [6].

Conclusions for the mobility of proteins

So we have the result that metHbH$_2$O and aquometmyoglobin (metMbH$_2$O) are both in a high-spin state at all temperatures. This is, what could have been expected from the similar heme environment. According to our interpretation, the difference between the two proteins is that the His-binding can take place in hemoglobin and is strongly hindered in myoglobin. Moreover, in hemoglobin the subunits have different binding characteristics and, what is the most surprising effect, even hemoglobin crystals behave very differently from a solution of hemoglobin. So we find very different behaviours in spite of very similar heme environments. Therefore it has to be concluded that groups at some distance from the heme must be displaced so that the reaction can take place. This confirms the result of earlier work that the reaction is accompanied by large displacements of several groups; to render the binding of the N_ε to the heme-ion possible, the E-helix has to move 0.2 nm towards the heme, as was found by a computer simulation [16]. Also the very slow reaction rates (or large activation quantities) give evidence of a large-scale motion coupled with the reaction. Thus, large thermal fluctuation of the tertiary structure of hemoglobin are necessary to render the reaction possible.

The reaction can therefore be understood as a probe for the study of molecular fluctuations of large amplitude. That this reaction is found in frozen solution – without a greater discontinuity in the reaction rates at the freezing point of water, shows that protein fluctuations of large amplitude are possible in a frozen solution and are obviously not much influenced by the phase of the bulk water. For a better understanding of the interaction between the protein fluctuations and the properties of the water, studies of the viscosity dependence of the reaction are in progress.

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