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Temperature-jump studies and polarized absorption spectroscopy of methemoglobin-thiocyanate single crystals

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Association equilibria and association kinetics of the thiocyanate binding reaction to methemoglobin in single crystals and solution are studied using temperature-jump technique and polarized absorption spectroscopy. Different kinetic constants are found for the reaction in solution and crystal phase for the α- and β-subunits of the methemoglobin tetramer. The reduction of the reactivity of the α- and β-subunits in crystalline phase is 6-fold and 2.4-fold, respectively, compared to the values found in solution. The intramolecular binding reaction of the N_ε of the distal histidine E7 which is observed in methemoglobin in solution cannot be detected in single crystals. Our results suggest that crystallization of hemoglobin has little influence on small-scale structural fluctuations which are necessary for ligands to get to the binding sites and large-scale structural motions are suppressed.

Introduction

The structures of a great number of crystalline proteins have been deduced by X-ray analysis (Protein Data Bank Brookhaven) and the first time-resolved X-ray crystallographic studies of the conformational change of a protein was recently published [1]. These studies give valuable insight into the structural and dynamical basis of protein function. However, the structures and dynamic properties in crystals may be influenced by a sum of different intermolecular interactions. Since these interactions change between crystal and solution state, the conformational properties and the dynamics of the proteins may also change. Thus, for a correct interpretation of solution state behavior it is necessary to correlate crystal phase and solution phase structures and dynamics. Furthermore, calculations of thermodynamic properties by means of molecular dynamics simulations start from the basis of the crystal coordinates [2]. A comparison of the results with the parameters determined from experiments including crystallized proteins will be of interest. Therefore, one of us has developed temperature-jump techniques which are applied to study the reaction kinetics in protein single crystals [3,4], and we discuss the thiocyanate binding reaction in methemoglobin single crystals in the present paper.

Several observations on hemoglobin and myoglobin indicate that structure and dynamics are different in crystal and solution phase. Azide binding to horse methemoglobin and myoglobin is up to 22-fold slower in crystals than in solutions [5,6]. Structure and kinetics of CO binding is altered in the crystalline state [7,8]. Spectra of polycrystalline methemoglobin with high spin ligands, F^- and H_2O, show distinct differences to the spectra of solved methemoglobin [9,10].

The reaction of thiocyanate with methemoglobin in solution shows biphasic kinetics [11,12] with different reaction rates for the α- and β-subunits. In the present paper the equilibrium constants and reaction rates of this reaction in methemoglobin single crystals are determined and compared to the values found in solution. The problem of ligand diffusion in the crystal is discussed in detail. The association equilibria and relaxation kinetics are detected by means of polarized absorption spectroscopy which allows the determination of transition moment directions in the heme absorption region and of changes of the relative orientations of the heme planes in the hemoglobin molecule due to the ligand binding to the heme iron.

Abbreviation: methb, methemoglobin.

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Materials and Methods

Preparation of methemoglobin crystals and solutions

The preparation of the oxyhemoglobin from fresh horse blood followed the methods of Benesch et al. [13]. The oxidation of the hemoglobin to methemoglobin (methb) was achieved by the addition of a 3-fold amount of $K_3(Fe(CN)_6)$. The sample was desalted by running through a column of Sephadex G-25. Single crystals were prepared by mixing the solution of methemoglobin with buffered ammonium sulfate solutions according to the method of Perutz [14]. Spectroscopy and temperature-jump measurements of the crystals were performed with ammonium sulfate replaced by 1.2 M Na_2SO_4.

Optical clean crystals were selected from the preparations and washed several times with 1.2 M Na_2SO_4 solution. The pH of this solution was adjusted to 7.0 with phosphate buffer. At a wavelength of 633 nm the maximum crystal extinction coefficients have values of 4000 M^{-1} cm^{-1}. The heme concentration in the crystal calculated from the dimensions of the unit cell [15] is about 0.038 M. A peak optical density of about 1 requires a crystals thickness on the order of 50 μm. Crystals of this size have lateral dimensions of about 200×200 μm^2.

Ligand binding kinetics in solution and in single crystals were performed in 1.2 M Na_2SO_4 and 0.5 M phosphate buffer, pH 7.0 ± 0.1, and $T = 20 \pm 0.2$°C unless otherwise stated. The heme iron concentration in the solution measurements was approx. 30 μM.

Temperature-jump apparatus and detection system

Temperature-jumps were achieved in the sample using two different heating techniques, heating by a laser beam and dielectric heating. The whole apparatus with experimental and theoretical considerations of the heating and detection systems is described in detail in [3,4]. In the first case the temperature rise in the sample is achieved by the inset of absorption of focused continuous radiation of a HeNe laser. The laser is simultaneously used as the detecting light source. The final temperature and steady state is reached when the heat dissipation in the sample equals the laser power absorbed by the crystal. In the experimental setup used here typical temperature-jump amplitudes are $\Delta T = 6$ K and the steady state is reached within a time of $\Delta t = 50$ ms. Thus, chemical relaxations with relaxation times between 100 ms and several 10^3 s can be studied using this technique.

In order to achieve shorter heating times dielectric heating by microwave absorption was applied [4]. The crystal is placed in a sample cell in the center of a tunable TE 101 cavity. An adjustable short circuit allows the cavity to be matched to the frequency of the microwave source, a low cost 2M186 magnetron operating at 2.5 GHz. The sample cell covers a volume of 2 mm^3 filled with the crystal and the covering Na_2SO_4 solution. Microwave pulses with pulse lengths of 50 μs deliver temperature rises of 1 K. The amplitudes of the temperature-jumps were calibrated utilizing the known temperature dependence of the pH of Tris buffer with litmus as an indicator. Heating pulses longer than 50 μs caused artefacts, most probably due to convection flows and movements of the crystal. For temperature-jumps less than 1 K the temperature was sufficiently constant for some hundred ms after the heating pulse and relaxations in the time range between 100 μs and 0.2 s can be studied using this dielectric heating technique. The application of a high power magnetron would further decrease the heating time. A HeNe laser operating at $\lambda = 633$ nm was used as detecting light source.

Ligand-binding kinetics in solution was determined with a temperature jump apparatus built according to the scheme of Eigen and DeMaeyer [16] equipped with an interference filter of $\lambda = 424$ nm.

The microspectrophotometer

Stationary crystal spectra were recorded using a home-made microspectrophotometer, built similar to the scheme given by Eaton and Hofrichter [17]. A 150 W halogen bulb serves as a light source. The beam is chopped by a revolving ten sector disk and passes a monochromator (Bausch and Lomb High Intensity Grating Monochromator). The wavelength drive consists of a stepping motor, which is coupled to the monochromator and controlled by a personal computer. After polarization by a calcite prism the light beam illuminates a field diaphragm. The image of the field diaphragm is focused within the boundaries of a uniform region of the sample, thus optically masking the observed field [17]. The sample, a methemoglobin single crystal, is contained in a 1-mm cuvette filled with buffered 1.2 M Na_2SO_4 solution and the desired amount of NaSCN. This cuvette is mounted on a rotating microscope stage. The crystal can be observed by a modified microscope as visual control. After correct adjustment the image of the crystal is reoriented onto a receiver diode (BPX 65). The diode signal is amplified by a lock-in amplifier and recorded after A/D conversion (Analog devices, 16 bit) in a personal computer. The light source was sufficiently stable so that a reference beam was not used. The intensities with and without sample, $I(\lambda)$ and $I_0(\lambda)$, are recorded in consecutive manner, the extinction $E(\lambda) = -\log\{I(\lambda)/I_0(\lambda)\}$ was calculated subsequently.

Theory

Polarized absorption technique

Dichroism is a property of well-ordered structures. Absorption of polarized light is maximized with the

incident light beam polarized parallel to the electric dipole transition moment and vanishes for perpendicular directions. In methb crystals dichroism appears due to the regular orientation of the porphyrine planes of hemoglobin, which produce the entire absorption spectrum in the visible region.

There are two hemes in each asymmetric unit of the crystal, one α- and one β-heme. The angles between the heme axes, denoted x and y within the heme plane and z perpendicular to it, and the crystal axes a, b and c are well known from X-ray data [18]. Our experiments have been carried out on the monoclinic (space group C2) form of methb, which grow in a platy habit flattened on (001). The (001) face contains the a and b axis of the crystal.

The crystal extinction coefficients with the incident light polarized parallel to the a or b axis, ε_a and ε_b, can be written in terms of the molecular extinction coefficients $\bar{\varepsilon}$ determined from solution measurements and the orientation of the heme groups relative to the crystal axes. For a perfect x,y-polarized transition ($\varepsilon_x = \varepsilon_y = 3/2\ \bar{\varepsilon}$, $\varepsilon_z = 0$) we have

$$\begin{aligned}\varepsilon_a &= \frac{3\bar{\varepsilon}}{4}(\sin^2 z_\alpha a + \sin^2 z_\beta a)\\ \varepsilon_b &= \frac{3\bar{\varepsilon}}{4}(\sin^2 z_\alpha b + \sin^2 z_\beta b)\end{aligned} \tag{1}$$

and for a perfectly z-polarized transition ($\varepsilon_x = \varepsilon_y = 0$, $\varepsilon_z = 3\bar{\varepsilon}$)

$$\begin{aligned}\varepsilon_a &= \frac{3\bar{\varepsilon}}{2}(\cos^2 z_\alpha a + \cos^2 z_\beta a)\\ \varepsilon_b &= \frac{3\bar{\varepsilon}}{2}(\cos^2 z_\alpha b + \cos^2 z_\beta b)\end{aligned} \tag{2}$$

Here $z_i k$ is the angle between the z-axis of the i-subunit within the unit cell and the k-axis of the monoclinic crystals. ε_x, ε_y and ε_z are the heme molecular extinction coefficients. The squared direction cosines for heme normals of horse methb obtained from X-ray data are given in Table I.

From Eqs. 1 and 2 and the values of the direction cosines (Table I) the polarization ratio, $PR = \varepsilon_b/\varepsilon_a$, may be calculated: $PR = 2.9$ for x,y-polarized transitions and 0.3 for z-polarized transitions. For methbH_2O (pH 7) the experimentally observed transitions are nearly completely x,y-polarized as it can be seen from the PR spectrum, $2.2 \leqslant PR \leqslant 2.5$ in the wavelength range 350 nm $\leqslant \lambda \leqslant$ 680 nm (Ref. 17 and cf. Fig. 3). The difference between the calculated value for the PR and the values determined from experiments is not elucidated yet.

TABLE I

Squared direction cosines for heme normals of horse methb calculated from X-ray data [17,18]

Heme	$\cos^2 za$	$\cos^2 zb$
α	0.7341	0.1949
β	0.7346	0.2572

The heme molecular extinction coefficients and consequently the crystal extinction coefficients change as a result of a chemical reaction of the heme iron with ligands. A different behavior in the change of ε_a and ε_b and consequently a change of the PR can be observed, if the ligand binding results in a reorientation of the heme planes with respect to the crystal axes or if a z-polarized absorption band is rised.

Diffusion of ligands and chemcial relaxation

The mother liquor covering the single crystal in the sample cell serves as a great buffer of ligands. The association or dissociation of ligands in the methemoglobin crystals due to the temperature-jump will lead to a concentration gradient of ligands between crystal and covering mother liquor. The influence of ligand diffusion and possible convection flows in the sample volume on the observed rates of the chemical process will be discussed in this section.

For a reaction scheme

$$A + L \rightleftarrows B \tag{3}$$

with the equilibrium constant

$$K = \frac{c_B}{c_A \cdot c_L} = \frac{k_1}{k_{-1}} \tag{4}$$

the change of the concentration of A near equilibrium, Δc_A, with time t due to the binding of the ligand L is given by the usual rate equation [19]

$$d\Delta c_A/dt = -\{k_1(c_L + c_A \cdot Q) + k_{-1}\} \tag{5}$$

In deriving this equation the temperature-jump is considered as a step function perturbation. The parameter Q allows for the diffusional process of the ligands between crystal and covering mother liquor and is given by

$$Q = (c(z', t) - c_f)/(c_i - c_f) \tag{6}$$

$c(z', t)$ is the ligand concentration in the crystal, whose initial concentration has been c_i, while its final concentration is c_f.

If the diffusion of the ligands is fast compared to the rate of change of Δc_A we have $Q \approx 0$. Integration of Eqn. 5 gives

$$\Delta c_A = \Delta c_A(t = 0) \cdot \exp(-t/\tau) \tag{7}$$

with

$$\tau^{-1} = k_1 c_L + k_{-1} \tag{8}$$

In the limit of very slow diffusion compared to the rate of change of Δc_A, we have $Q \approx 1$, and

$$\tau^{-1} = k_1 \cdot (c_L + c_A) + k_{-1} \tag{9}$$

If the chemical relaxation rate is comparable to the diffusion rate the time dependence of Q has to be taken into account in the integration of Eqn. 5. To get an idea of the order of magnitude of the diffusion rates the behavior of Q with time is calculated for the different heating techniques and boundary conditions in the next section.

Ligand diffusion in the case of temperature-jump by dielectric heating

For the following estimations the crystals are approximated by thin plates of thickness $2h$ and infinite lateral extensions in the a,b-plane. One-dimensional diffusion along the axis perpendicular to the a,b-plane, defined as z'-axis, is considered. The value of the diffusivity, D, is chosen according to the value found for SCN^- ions in water, $D = 1.3 \cdot 10^{-3}$ mm^2 s^{-1}. This approximation may be justified due to the high water content of the crystals of 53% [15], the resulting diffusion rates will be considered as upper bounds. We have to consider two different boundary conditions for the ligand concentration near the surface of the crystal. In the absence of any convection flows in the solution covering the crystal, concentration gradients are neutralized by diffusion in the whole sample volume. The dimensions of the volume of the covering mother liquor are much greater than the crystal thickness $2h$, so that the ligand concentration far apart from the crystal surface, $c(z' \gg h)$, may be regarded as time-independent.

The solution of the diffusion equation in this case is given by [20]

$$Q(z', t) = 0.5\left\{\operatorname{erf}\left(\frac{h+z'}{2\sqrt{Dt}}\right) + \operatorname{erf}\left(\frac{h-z'}{2\sqrt{Dt}}\right)\right\} \tag{10}$$

with the notation

$$\operatorname{erf} x = 2/\sqrt{\pi} \int_0^x d\xi \cdot \exp(-\xi^2)$$

The behavior of Q averaged over the whole crystal thickness $2h$ is shown in Fig. 1 for $h = 20$ μm. After a delay of 400 ms the difference between the concentrations in the interior of the crystal and in the covering mother liquor is reduced to 45%.

If convection flows in the covering mother liquor ensure that the concentration of the ligand at the crystal surface, $c(z' = h)$, is constant with time, the behavior of Q is given by [20]

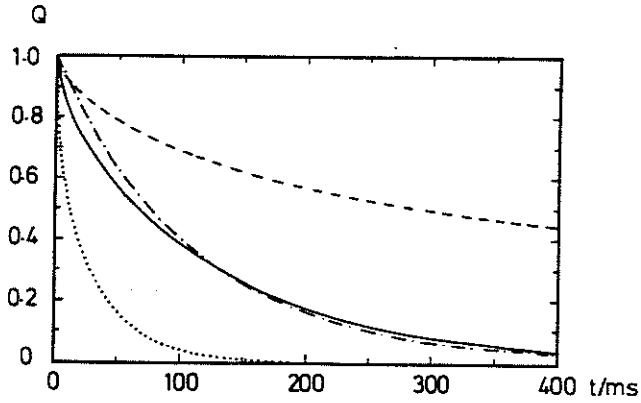

Fig. 1. The change of the normalized concentration difference between crystal and covering mother liquor, Q, with time t due to diffusion. Q is averaged over the whole crystal thickness of 40 μm. Calculations have been performed with (———) and without (– – –) consideration of convection flows in the covering mother liquor for the case of microwave heating. A least squares fitting of an exponential to $\bar{Q}(t)$, e.g., in the case of convection flows (·–·–·), shows that the characterization of the diffusion rate by the time constant of an exponential is justified. Temperature-jump by absorption of laser radiation (·····) with a beam diameter of 22 μm leads to comparably fast equilibration of the ligand concentration.

$$Q(z', t) = 4/\pi \sum_{n=0}^{\infty} \frac{(-1)^n}{(2n+1)} \exp\left(\frac{-D(2n+1)^2\pi^2 t}{4h^2}\right) \cdot \cos\left(\frac{(2n+1)\pi z'}{2h}\right) \tag{11}$$

The relation of Q to time t according to Eqn. 11 averaged over the whole crystal thickness $2h$ is also shown in Fig. 1. Now the concentration difference is less than 3% after 400 ms.

A single exponential fitted to the time courses of Q is used to characterize the diffusion rates. The example displayed in Fig. 1 shows that this may be regarded as an appropriate way of characterization. We get a relaxation time τ_D of 500 ms and 110 ms in the cases with and without convection flows, respectively. Evidence for convection processes have been observed in our experiments. Under certain conditions inhomogeneous heating due to the finite penetration depth of the microwaves into the sample results in temperature gradients. Thus, for large temperature-jump amplitudes movements of the crystals have been detected, probably caused by convection flows in the mother liquor.

The value of D for the diffusion of SCN^- ions in bulk water is used as a first approximation in the above calculations. A decrease of D due to restricted diffu-

sion of the ligands in the crystal will further increase the diffusion relaxation time. The values of τ_D given above are, therefore, regarded as a lower bound. Thus, if we deal with chemical reactions with relaxation times less than 100 ms Eqns. 9 is a good approach for the behavior of the apparent relaxation rates with ligand concentration.

Ligand diffusion in the case of temperature-jump by irradiation with a laser beam

When the temperature-jump is achieved by laser beam irradiation the volume of the irradiated crystal element may be approximated by a sphere of radius w, where w is the radius of the laser beam at the e^{-2} points [3]. This approximation is valid, if the absorption coefficient is sufficiently large so that the main part of radiation energy is absorbed within a distance w in the crystal. For methemoglobin single crystals this requirement is fulfilled for $w \geqslant 20$ μm and the incident light beam polarized parallel to the b-axis or $w \geqslant 40$ μm and the incident light beam polarized parallel to the a-axis. The average normalized concentration difference is now given by [20,3]

$$\bar{Q} = \frac{\bar{c} - c_f}{c_i - c_f} = \frac{6}{\pi^2} \sum_{\nu=1}^{\infty} \frac{1}{\nu^2} \exp(-\nu^2 \pi^2 Dt / w^2) \tag{12}$$

A least-squares fitting of an exponential to the time course of $\bar{Q}$ with $w = 22$ μm yields the relaxation time of diffusion, $\tau_D = 40$ ms. In the experimental section this temperature-jump method is used when the chemical relaxation times expected are much greater than τ_D. In this case Eqn. 8 is appropriate and the kinetic constants are calculated from the slope and the intercept of the plot of the relaxation rates, τ^{-1}, versus ligand concentration, c_L.

Relaxation amplitudes

The relaxation amplitude

$$X = \Delta E/(E \cdot \Delta T) = \frac{1}{E} \cdot \frac{\Delta E}{\Delta K} \cdot \frac{\Delta K}{\Delta T} \tag{13}$$

is deduced for the general case that the association constants and kinetic constants of the ligand-binding reaction to the α- and β-subunits are different. The ratio $\Delta K/\Delta T$ is calculated from

$$K = \exp(-\Delta H^0/RT + \Delta S^0/R) \tag{14}$$

and yields

$$\Delta K/\Delta T = K \cdot \Delta H^0/RT^2 \tag{15}$$

where the symbols have their usual meanings. The concentration of the ligand L, c_L, does not change since the greater volume of mother liquor covering the crystal serves as a buffer of L, and we shall determine the amplitudes in a time interval where the concentration gradients have been neutralized by diffusion. The overall extinction of the crystal E_i with the incident light polarized parallel to the crystal axis, $i = a$, b, is composed of the extinctions of the liganded and unliganded α- and β-subunits and is calculated in terms of c_L and the equilibrium constants of the reaction, K_α and K_β:

$$E_i = \varepsilon_{i,A} \cdot c_0 \cdot d \cdot \{(V \cdot K_\alpha \cdot c_L + 1)/(1 + K_\alpha c_L) + (V \cdot K_\beta \cdot c_L + 1)/(1 + K_\beta \cdot c_L)\} \tag{16}$$

where $V = \varepsilon_{i,B}/\varepsilon_{i,A}$, $\varepsilon_{i,B}$ and $\varepsilon_{i,A}$ are the extinction coefficients of the liganded and unliganded hemes, the respective values are assumed to be equal for α- and β-subunits. c_0 is half of the concentration of the hemes in the single crystal and d is the crystal thickness. The derivation with respect to K_β, for example, and division by E_i yields

$$\Delta E_i/(E_i \cdot \Delta K_\beta) = \frac{c_L(V-1)/(1 + K_\beta c_L)}{(V \cdot K_\beta \cdot c_L + 1) + (V \cdot K_\alpha \cdot c_L + 1)(1 + K_\beta \cdot c_L)/(1 + K_\alpha \cdot c_L)} \tag{17}$$

If the equilibrium constants K_α and K_β are well separated, the temperature-jump experiment can be performed in concentration ranges of c_L where $K_\alpha c_L$ is either much less or much greater than unity and K_α can be eliminated from Eqn. 17. Multiplication of Eqn. 17 with Eqn. 15 yields the desired relaxation amplitude.

Results

Association equilibria

The value of the polarization ratio, PR, was determined for 48 crystals at $\lambda = 633$ nm, varying the thiocyanate concentration, [SCN^-], between 0–0.128 M. Results are shown in Fig. 2. The PR decreases with increasing [SCN^-]. Data for thiocyanate concentrations much greater than 0.1 M could hardly be obtained because of the reduced stability of the methemoglobin single crystals. The ligand binding reaction of thiocyanate in solution shows biphasic kinetics, requiring two association constants to adequately describe the results [11,12]. Thus, the dependence of PR on two association constants, K_α and K_β, and ligand

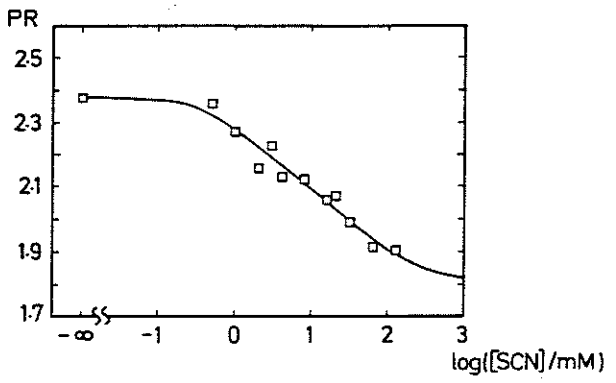

Fig. 2. The polarization ratio, PR, of methb as a function of the thiocyanate concentration, [SCN]. The solid line is a least-squares fitting of Eqn. 18 to the experimental data points with parameters $K_{\alpha,\beta}$ shown in Table II.

concentration c_L was calculated:

$$PR = \left\{ PR^{(\infty)} \left(\frac{K_\beta c_L}{1+K_\beta c_L} + \frac{K_\alpha c_L}{1+K_\alpha c_L} \right) + PR^{(0)} \frac{\varepsilon_a^{(0)}}{\varepsilon_a^{(\infty)}} \left(\frac{1}{1+K_\beta c_L} + \frac{1}{1+K_\alpha c_L} \right) \right\} \times \left\{ \left(\frac{K_\beta c_L}{1+K_\beta c_L} + \frac{K_\alpha c_L}{1+K_\alpha c_L} \right) + \frac{\varepsilon_a^{(0)}}{\varepsilon_a^{(\infty)}} \left(\frac{1}{1+K_\beta c_L} + \frac{1}{1+K_\alpha c_L} \right) \right\}^{-1} \quad (18)$$

$PR^{(\infty)}$ and $PR^{(0)}$, $\varepsilon_a^{(\infty)}$ and $\varepsilon_a^{(0)}$ are the polarization ratios and the extinction coefficients for methbSCN$^-$ and methbH$_2$O with the incident light polarized parallel to the a-axis of the crystal, respectively. The value of $PR^{(0)}$ was determined to 2.38 ± 0.01, and the extinction coefficient ε_a did not change significantly with increasing thiocyanate concentration so that we set $\varepsilon_a^{(0)}/\varepsilon_a^{(\infty)} = 1$. The fitting procedure of Eqn. 18 with the parameters, $PR^{(\infty)}$, K_α and K_β, to the experimental data points yields two well separated values of the equilibrium constants which we refer to the binding of the ligand to the α- and the β-heme, respectively. The behavior of Eqn. 18 is shown in Fig. 2, the values of K_α and K_β are given in Table II. (The lower value of K is attributed to the α-subunits in accordance with the results found in solution, see below.)

A decrease of PR due to the thiocyanate binding may be the consequence of a reorientation of the heme plane or the rise of a z-polarized absorption band. To decide which of these mechanisms is the dominant process the polarization ratio is studied as a function of the wavelength λ. A reorientation of the heme plane would reduce the value of PR independent of λ, whereas a z-polarized transition should obey a distinct

TABLE II

Association constants, $K_{\alpha,\beta}$, (T = 293 K, pH 7.0, 1.2 M Na_2SO_4) for the thiocyanate binding reaction to methemoglobin in single crystals and in solution, determined by different methods

Phase	Solution	Crystal			
method [a]	k_1/k_{-1}	PR	$X_{\mu s}$	X_{ms}	$k_{1\alpha}/k_{-1\alpha}$
$\log(K_\beta/M^{-1})$	1.9±0.1	2.5±0.2	(2.8±0.3)	2.3±0.3	–
$\log(K_\alpha/M^{-1})$	1.5±0.1	1.2±0.8	(1.2±1.1)	–	1.5±0.1

[a] k_1/k_{-1}: $K_{\alpha,\beta}$ determined from kinetic data according to Eqns. 4 and 8;
PR: $K_{\alpha,\beta}$ determined from PR vs. [SCN] measurements according to Eqn. 18.
$X_{\mu s}$: $K_{\alpha,\beta}$ determined from the fitted titration curve to the behavior of the amplitude of the μs-relaxation vs. [SCN].
X_{ms}: K_β calculated from the amplitudes of the ms-relaxation according to Eqns. 17 and 15.

dependence on λ and, therefore, the change of PR will not be uniform. The relations of PR to λ for methb single crystals covered with a buffered Na_2SO_4 solution (pH 7), containing 0 and 64 mM SCN are shown in Fig. 3. The value of PR of the methbH$_2$O crystals varies between 2.2–2.5 in the wavelength range 480 nm $\leqslant \lambda \leqslant$ 650 nm. These values are in good agreement with those reported for methb in the Soret region, $PR = 2.47$ [17]. The slight deviations of PR from a constant value may be due to transitions that are not x,y-polarized. The variation of PR with λ for the methbSCN sample is by far more pronounced. Thus, this spectrum can be explained if we assume the existence of a z-polarized absorption band. An additional reorientation of the heme plane, however, cannot be excluded.

Association kinetics

Thiocyanate binding kinetics in solution. The relaxation transients of the SCN-binding kinetics in 1.2 M Na_2SO_4 solution (pH 7.0), are well fitted by a superposition of two exponentials. No deviations from linear dependency of the two relaxation rates on the ligand

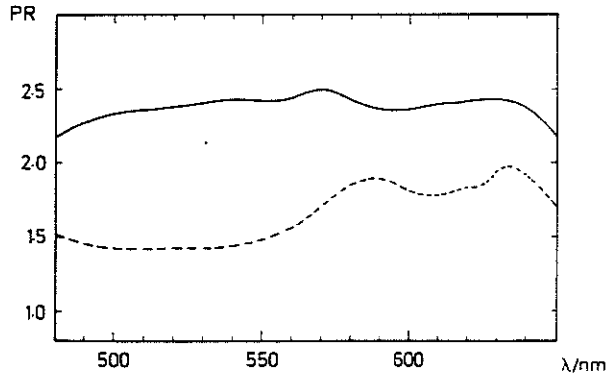

Fig. 3. The dependence of the polarization ratio, RR, on wavelength λ for methbH$_2$O (———) and methbSCN (– – –) (pH 7.0).

TABLE III

Kinetic constants for the SCN-binding reaction to methemoglobin in solution and in single crystals, T = 293 K, pH = 7.0, 1.2 M Na_2SO_4

	$k_{1\alpha}$ / $(M^{-1}\,s^{-1})$	$k_{-1\alpha}$ / s^{-1}	$k_{1\beta}$ / $(10^3\,M^{-1}\,s^{-1})$	$k_{-1\beta}/s^{-1}$
Solution	240±20	7.5±1.5	2.2±0.1	30 ±10
Crystal	30± 2	1.3±0.2	0.9±0.1	4.5± 2.5

concentration are observed in the range 2 mM $\leqslant$ [SCN] $\leqslant$ 100 mM. From linear-least squares regression analysis we calculated the kinetic constants k_1 and k_{-1} and equilibrium constants $K = k_1/k_{-1}$ for the observed reactions, the corresponding values are shown in Table II and Table III. Our results of a biphasic reaction are in agreement with the results of other authors [11,12]. The velocity constant for this reaction with the isolated ferric α- and β-subunits was different, being higher for the β-chains [11]. Therefore, we consider the fast and slow relaxation as the ligand reactions with the β- and α-chains respectively.

Thiocyanate binding kinetics in methemoglobin single crystals. Different relaxations on different time scales can be observed in methbSCN single crystals. Typical transients for $\Delta E/(E\Delta T) = X$ are shown in Fig. 4. The transients recorded with the incident light polarized parallel to the a- and b-axes of the crystal differ significantly. The temperature-jump is accompanied by a decrease of the extinction, the time course of this μs-relaxation cannot be resolved and a relaxation rate greater than $10^5\ s^{-1}$ has to be assumed. The dielectric properties of the crystals and the covering mother liquor are different leading to different temperatures in the crystal and the covering mother liquor during the dielectric heating process [4]. At the end of the microwave pulse the crystals cool down to the temperature of the covering mother liquor, these temperature relaxations depend on the crystal thickness and have half-lives of between 1–4 ms for the crystals used here. The extinction change due to the μs-effect follows this temperature relaxation without any delay. A second relaxation with relaxation times in the ms time range can be resolved in the transient of $\Delta E_b/E_b$. The amplitude $\Delta E_a/E_a$ of this relaxation with the incident light polarized parallel to the *a*-axis is by far less and cannot be separated from the background. Using the laser heating technique a third relaxation with relaxation times in the s-range can be resolved, an example is given in Fig. 4c. In the following sections we shall deal with these three relaxations and show that the information about the thiocyanate binding kinetics can be extracted from the amplitudes and time courses of the ms- and s-relaxation.

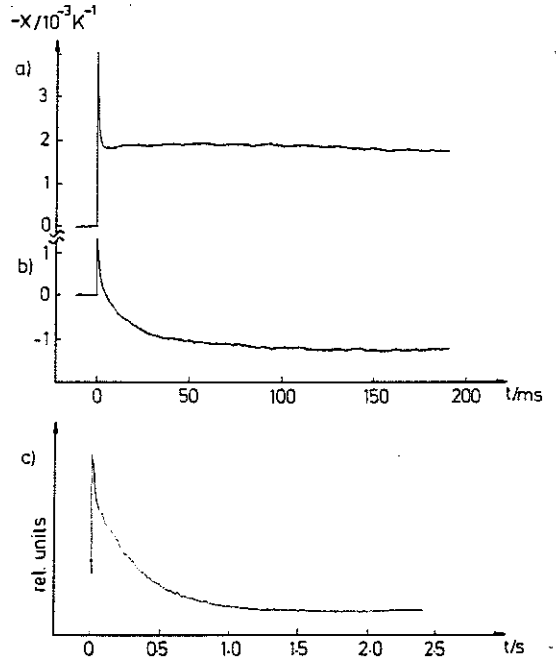

Fig. 4. Relaxation transients of methb single crystals after a temperature-jump at $T = 293$ K, $\Delta T = 1$ K. (a) Extinction exchange $X_a = \Delta E_a/(E_a\Delta T)$ recorded with the incident light beam polarized parallel to the *a*-axis of the crystal; dielectric heating, [SCN] = 3 mM. (b) Experimental conditions as in (a) with the incident light beam polarized parallel to the *b*-axis of the crystal. (c) Temperature-jump by absorption of a laser beam and detection of X_b, [SCN] = 30 mM.

The μs-relaxation

The amplitudes of the μs relaxation, $\Delta E_a/(E_a\Delta T) = X_a$ and $\Delta E_b/(E_b\Delta T) = X_b$, are identical within experimental error for crystal samples without SCN ligand added, $X_{a,b} = -(2.15 \pm 0.20)\cdot 10^{-3}\ K^{-1}$. A similar relaxation is found for methbH_2O in solution with the amplitudes depending on the detecting wavelength λ [21]. For $\lambda = 633$ nm we have $X = -(2.2 \pm 0.2)\cdot 10^{-3}\ K^{-1}$ reflecting similar behavior for methbH_2O in crystal and solution phase. Relaxation times could not be resolved in the present and in former studies and fast multistep-equilibria cannot be excluded [21]. After addition of SCN the amplitude X_a remains unchanged within experimental error. However, the relaxation amplitude $|X_b|$ decreases monotonically with increasing SCN concentration as shown in Fig. 5. This behavior does not reflect the binding kinetics of the ligand because in this case we expect a bell-shaped dependency of X on [SCN^-] (cf. Eqn. 17). A titration curve with two equilibrium constants, $K_{\alpha,\beta}$, and the parameter $X_b([SCN] \to \infty)$, fits the experimental data well, the decrease of the amplitude X_b is linearly correlated with the amount of water ligands which are replaced by thiocyanate ions. The two constants $K_{\alpha,\beta}$ determined from the fit are shown in Table 2. They coincide with the values determined from the PR measurements. In spite of the large error estimates on K_α we regard this result as a second hint that the μs-relaxation does not reflect the ligand binding kinetics. The temperature

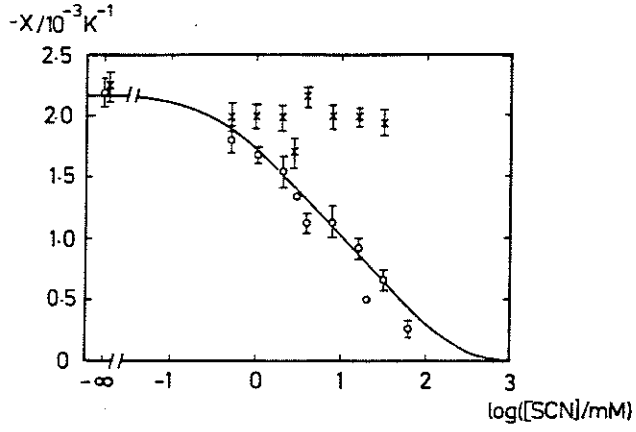

Fig. 5. Relaxation amplitudes X_a (×) and X_b (○) of the μs-relaxation as a function of thiocyanate concentration, [SCN]. The line is a least-squares fitting of a titration curve with two equilibrium constants to the experimental data points of X_b.

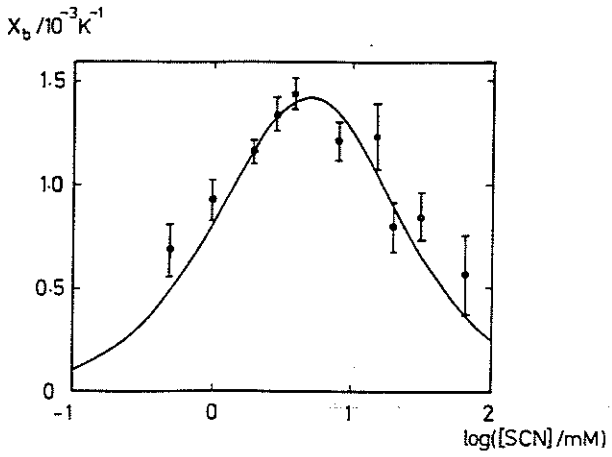

Fig. 6. The dependence of the relaxation amplitudes of the ms-relaxation with the incident light beam polarized parallel to the crystal *b*-axis, X_b, on thiocyanate concentration. The bell-shaped curve shows the behavior of Eqns. 15 and 17 with the parameter K_β given in Table II.

dependence of this relaxation found in solution [21] and the different behavior of X_a and X_b give evidence that we are dealing with a fast multistep mechanism and we do not try to further interpret the underlying mechanisms. The study of the two remaining relaxations in the next sections will completely characterize the thiocyanate binding kinetics and equilibria.

The ms-relaxation

The time course of the ms-relaxation could be resolved with concentrations of SCN between 0.5–64 mM with the incident light polarized parallel to the β-axis of the crystal. The amplitude X_a of this relaxation is again less than $5 \cdot 10^{-4}$/K and cannot be resolved from the background. However, the amplitudes X_b depend significantly on [SCN] as shown in Fig. 6. We get a bell-shaped curve with its maximum for a thiocyanate concentration of about 4 mM. The fit of Eqns. 17 and 15 to the experimental data points shows good agreement with the experimentally determined behavior and delivers the parameters ΔH^0 and K. Here we set $V = \varepsilon_{b,A}/\varepsilon_{b,B} = 0.76$. The equilibrium constant determined from this kinetic experiment is shown in Table II and coincides with the values of K_β determined from the studies of *PR*([SCN]). From this result we conclude that the ms-relaxation reflects the kinetics of the ligand binding reaction to the β-hemes. The value of ΔH^0 is calculated from the fit to (-34 ± 4) kJ/mol which is in good agreement with the value found for this reaction in solution, $\Delta H^0 = -31.5$ kJ/mol [11].

The relaxation rates, τ_β^{-1}, calculated from the time course of X_b are shown in Fig. 7. Since the apparent concentration of hemoglobin in single crystals, c_0, is not known we renounce a plot of the rates vs. free ligands and free binding sites and display the values of the rates versus ligand concentration, c_L. The thicknesses of the single crystals used in this experiment range from 40 to 100 μm. Hence, the values of τ_β are much less than the lower bounds of the relaxation times of the diffusional process of the ligands in the crystals (cf. Fig. 1) and we have to take Eqn. 9 to determine the velocity constant k_1:

$$\tau_\beta^{-1} = k_{1\beta} \cdot (c_L + c_0/(1 + K \cdot c_L) + 1/K) \qquad (19)$$

where $c_0 = c_A + c_B$. To reduce the number of parameters we set K to the value found for K_β from the studies of the PR behavior and relaxation amplitudes (cf. Table II), $K_\beta = 200\ M^{-1}$ and fit $k_{1\beta}$ and c_0. The behavior of a least-squares fitting of Eqn. 19 to the

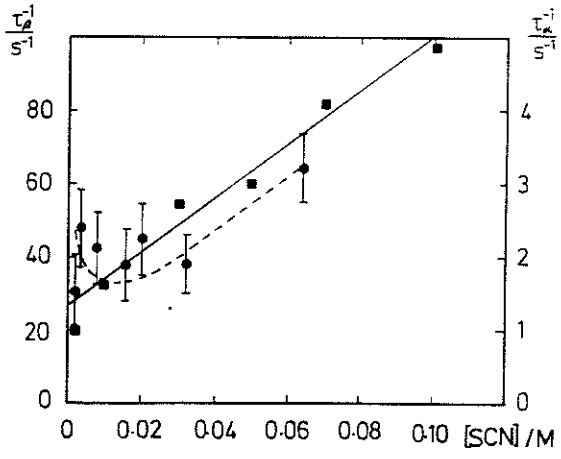

Fig. 7. Relaxation rates τ_α^{-1} (■) and τ_β^{-1} (●) of the thiocyanate binding reaction to methb single crystals. The solid line is calculated by a linear least-squares regression analysis to the experimental data points of τ_α^{-1} according to Eqn. 8. The broken line is a least-squares fitting of Eqn. 19 to the experimental data points of τ_β^{-1}.

experimental data points is shown in Fig. 7. The graph deviates from linear dependency for small ligand concentrations due to the high hemoglobin concentration in the crystals (cf. Eqn. 19) and is in agreement with the experimental data. The value of c_0 results to (60 ± 16) mM which agrees with the concentration of heme iron of the β-subunits with regard to the amount of free water in the crystals. The value of $k_{1\beta}$ for $T = 293$ K is given in Table III.

From the measurement of the temperature coefficient of the relaxation rates we calculate the energy of activation of this reaction, $\Delta H^{\ddagger} = (73 \pm 6)$ kJ/mol. This value is too large to account for a diffusion limited relaxation, for which we would expect $\Delta H^{\ddagger} = 26$ kJ/mol. Hence, the observed relaxation rates are due to the association of the ligand and there is no evidence for a contamination of the results by systematic errors due to ligand diffusion.

The s-relaxation

The study of the transients for times longer than 200 ms resolves a third relaxation with relaxation times between 200 ms and 1 s depending on thiocyanate concentration. Fig. 4c shows a plot of X_b vs. time of a methb single crystal with 30 mM SCN^-. For $t > 0.05$ s the experimental data points are excellently fitted by a single exponential over an amplitude range of about two decades (not shown). For $t < 0.05$ s the data points deviate from this exponential. These deviations are due to the ms-relaxation discussed in the preceding section.

The relaxation rates, τ_α^{-1}, obtained for the s-relaxation are shown in Fig. 7 as a function of thiocyanate concentration, c_L. The relaxation is not interfered by the diffusional process in this experiment since the relaxation times are at least 10-fold the typical diffusional time constants. According to Eqn. 8, which has to be applied in this limit of diffusion and relaxation rates, the chemical relaxation rates, τ_α^{-1}, appear to depend linearly on the ligand concentration, c_L. The values for the rate constants obtained from the graph, Fig. 7, are given in Table III and the value of the resulting $K = k_1/k_{-1}$ is shown in Table II. Good agreement is obtained with the values of K_α calculated from *PR* studies and, thus, we associate the present s-relaxation process with the ligand binding reaction to the α-hemes.

Discussion

Temperature-jump studies of the thiocyanate binding reaction to methemoglobin in single crystals with volumes less than $2 \cdot 10^{-3}$ mm^3 and in solution have been performed. Temperature and ligand diffusion in the crystal have been taken into account in the analysis of the experimental data. The thiocyanate binding reaction is at least biphasic and the results suggest that there is one fast reaction in which the ligand does not participate. We have analyzed the biphasic reaction on the assumption of two heme types which react independently and with different reactivities. The assumption is supported by the observation that the time course of the binding of SCN to methemoglobin in solution is heterogeneous (Refs. 11, 12, and this paper). Correspondingly, the velocity constant for this reaction with the isolated ferric chains was different in solution, being higher for the β-chains [11]. We associate the ms-effect and s-effect observed in the single crystal relaxation transients with the reaction of the β-hemes and the α-hemes, respectively. A comparison of kinetic and thermodynamic results (cf. Table II) yields good agreement so that there is no reason to treat the data in a manner other than that used here.

Comparison of the thiocyanate – methemoglobin reaction equilibrium and kinetics in crystal and solution phase

The equilibrium constants determined for the reaction of the α-chains in crystal and solution are shown in Table II and appear to be identical within experimental error. However, the respective values for the reaction of the β-chains seem to indicate an enhanced affinity of methemoglobin to thiocyanate in the crystalline state. This may be regarded as a first hint that structural differences between solution and crystal phase cannot be excluded.

The velocity constants found for the ligand binding to the α- and β-subunits in the crystals are significantly less than the values found in solution (cf. Table III). Hence, the ligand association reaction to methemoglobin is altered by crystallisation, the association (on) rates of this reaction with the α- and β-subunits are reduced by a factor 6 and 2.4, respectively, compared to the values found in solution. These observations are in agreement with the results of the reactions of suspensions of crystals of horse hemoglobin with azide [6]. The crystalline material reacted with azide in a biphasic process, the steps of which are 2.5- and 22-times slower than the monophasic reaction found for the soluble methemoglobin in 40% ammonium sulfate solution. The authors [6] concluded that intermolecular contacts decreases the accessibility of one pair of hemes with respect to the other pair. Later experiments on isolated ferric chains and the methemoglobin tetramer [11] have shown that the time course of the combination with azide is also heterogeneous in solution; correspondingly, the velocity constant for the combination of this ligand with the isolated ferric chains is different, being higher for the β-chains. Therefore, the observed factors of reactivity reduction, 2.5 and 22, are related to the ratios $k_{1\beta}^{\text{solution}}/k_{1\beta}^{\text{crystal}}$ and $k_{1\beta}^{\text{solution}}/k_{1\alpha}^{\text{crystal}}$, respectively, and no data are available in the literature to deduce the value of $k_{1\alpha}^{\text{solution}}/k_{1\alpha}^{\text{crystal}}$. The ligands used

in [6] and in the present studies are of similar dimensions and $k_{1\alpha}^{solution}/k_{1\beta}^{crystal}$ found in our experiments for the thiocyanate binding reaction agrees well with that given in [6] for the ~~oxide~~ binding reaction. Our results suggest that crystallization influences the properties of the α-subunits to a greater extent than those of the β-subunits.

X-ray analysis shows that the distal histidine (E7) blocks the access to the heme pocket [22]. Ligands cannot leave or enter unless the side chain of the distal histidine swings out of the way, which it can do only by elbowing the helix E away from the heme. Thus, ligand binding relies on the dynamic of the globin [23]. From O_2 binding kinetics in native hemoglobin and hemoglobin where the distal histidine is replaced by glycine it is concluded that histidine E7β must be swinging in and out at least 10^9 times per second, while in the α-subunits that rate appears to be about a 100-times slower [23]. These dynamic properties may be restricted in hemoglobin single crystals due to intermolecular interactions and greater rigidity of the protein structure. Our results show that these restrictions are more pronounced for the α-subunits.

The relaxation transients of methbH_2O and methb-SCN following a temperature-jump have been studied in a time range between ~ 100 μs and 100 s. There is no evidence for other reaction equilibria than those presented in the preceding sections. Especially a relaxation in the ms-range which is found in methH_2O in solution even in the frozen phase [21,24,25] cannot be detected in methbH_2O single crystals. The corresponding reaction has been attributed to the intramolecular binding of the N_ε of the distal histidine E7 to the heme iron and requires large scale movements of parts of the E-helix of about 0.2 nm [24]. We conclude that these large-scale fluctuations are suppressed in the crystal phase. However, the factor of the overall decrease of the association rates for the thiocyanate reaction in methemoglobin single crystals is less than one order of magnitude for both α- and β-subunits compared to the values found in solution. This is an indication for the hardly reduced ability of the crystallized protein to perform small scale structural fluctuations.

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