THERMAL FLUCTUATIONS OF LARGE AMPLITUDE IN THE TERTIARY STRUCTURE OF METHEMOGLOBIN

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1. Introduction

Investigations on the dynamics of folded proteins are of actual interest in the context of the problem of structure and function of these molecules [1]. We present here some results concerning the dynamics of the heme region of methemoglobin (MetHb) dimers at pH 6, T = 5–20°C in aqueous solution.

The results are based on the interpretation of our measurements in terms of an intramolecular reaction equilibrium characterized by binding of imidazole (Im), His E7 to the heme iron (Fe). This equilibrium was postulated first in [2,3]. It should be influenced by structural changes of the heme region, and we consider it as a test reaction for the investigation of thermal fluctuations in the geometry of that region.

In [4] and by new arguments in this paper it is shown that the equilibrium mentioned exists and is connected with the 'ms-temperature jump relaxation'. Therefore the T-jump method [5] seems to be suitable to study this equilibrium which can be separated quantitatively from other reactions occurring in the protein [6].

Comparison of data from T-jump and optical spectroscopy enables us to rule out one of two conceivable (and in [2] resp. [3] postulated) reactions in the heme pocket which both lead to a bond Im, His E7-Fe, but should be governed by tertiary fluctuations in a different manner.

Considering in addition X-ray data of the geometry of the heme pocket, one finds that the reaction observed here can take place only if the tertiary structure in the heme region carries out thermal fluctuations with amplitudes of 1–2 Å. Furthermore it is shown that in the intramolecular equilibrium under consideration an enthalpy-entropy overcompensation takes place such that in this case energy refinement calculations would lead to paradoxical results.

Perutz [7] has pointed at the special importance of His E7 in connection with the oxygen binding in myoglobin (Mb) and hemoglobin (Hb). In the case of Mb the O_2 cannot get into the heme pocket if His E7 does not move from its X-ray position. It should be considered whether the fluctuations of tertiary structure observed here play a role in this context.

2. Materials and methods

2.1. *MetHb preparations*

Oxyhemoglobin was prepared from fresh human blood by the method of Benesch et al. [8], stripped of organic phosphates, freed from Cl^- ions, and oxidized to MetHb with $K_3Fe(CN)_6$. All solutions were used within 3 days after preparation. The final solutions were some 10^{-5} M (heme), phosphate buffer, pH 6, ionic strength 0.1, where 80% hemes are in dimers. Details of the T-jump apparatus built will be published elsewhere.

2.2. *Determination of thermodynamic parameters*

In accordance with [4,6] the reaction underlying the ms-relaxation is described formally by the scheme

$$A \underset{k_{off}}{\overset{k_{on}}{\rightleftarrows}} B \; ; K = [B]/[A] = \exp -(\Delta H - T\,\Delta S)/RT \quad (1)$$

Then the time course of the optical extinction E after a T-jump $T_O \rightarrow T_O + \Delta T = T$ is given by

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$$E(t) = E(0) + \Delta E\,\{1-\exp(-t/\tau)\} \qquad (2)$$

which indeed was observed throughout all experiments. As described in [9], we have as measured quantity $\Delta E/E\ \Delta T = X(T, \lambda)$. Introducing additionally the derivative $Y = d\ln|X|T^2/d(1/T)$, which may be obtained as slope of a plot $\ln|X|T^2$ vs. 1/T, we get the rigorous relations:

$$\Delta H = RY\frac{(K+1)(K+V)}{K^2-V} \qquad (3)$$

$$K = \frac{(1-V)Y}{2XT^2} + \sqrt{\left[\frac{(1-V)Y}{2XT^2}\right]^2 + V} \qquad (4)$$

$V = \epsilon_A/\epsilon_B$ means the ratio of the molar extinction coefficients of A and B. If V is known, one can determine the thermodynamic data in Eqn. 1 from the measured function $X(T)$. In the limits $K \lesseqgtr 1$ follows from Eqn. 3 the simple and symmetrical expression $\Delta H = \mp RY$.

3. Results and discussion

3.1. *Thermodynamic data of the reaction investigated*

We obtained here the linear relation $\ln|X|T^2 = a/T + b$ with $a = (7693 \pm 620)\,K$ and $b = -23.9 \pm 2.2$ in the temperature range $T = 5$–$20°C$; $\lambda = 420$ nm.

Thus in our case $Y = a$ holds. For calculation of K from Eqn. 4 we use $V = 0.68$, ϵ_A and ϵ_B taken from [2,10]. The Van 't Hoff plot then leads to $\Delta H = (-64.5 \pm 5)$ kJ/mol and $\Delta S = (-275 \pm 20)$ J/mol K.

The small values of $K(T)$ calculated, for instance $K_{4°C} = 0.0055$ and $K_{20°C} = 0.0013$ indeed justify the approximation $K \ll 1$ which in [4] has been assumed and is proved here. Since $K \ll 1$, we also obtain directly and independently of V, $\Delta H = -RY = (-64.0 \pm 5)$ kJ/mol in agreement with the above rigorous calculation. Hence the reaction is strongly exothermic, the small values of K are due to the overcompensating entropy contribution. The kinetic results can be formally expressed by

$$1/\tau = kT/h \exp -(\Delta H^{\ddagger} - T\,\Delta S^{\ddagger})/RT \text{ with}$$

$$\Delta H^{\ddagger} = (51.6 \pm 9.3)\ \text{kJ/mol}$$

and $\Delta S^{\ddagger} = (-13.5 \pm 32)$ J/mol K.

3.2. *Nature of the ms-relaxation*

After [6] the rate of the ms-relaxation depends little on concentration and even on dimerisation of the MetHb molecules. Furthermore as shown in [4,6,11] the reaction is connected with a spin change of Fe. It was therefore concluded that the underlying reaction is intramolecular and takes place inside the heme pocket.

Now there are two reaction models discussed in the literature, which describe intramolecular reactions inside the heme pocket and are coupled with a high-to-low spin transition of Fe. In model I [2] Im, His E7 binds directly to Fe, the ligand H_2O (dominating at pH 6) is displaced. The essential step in model II [3] is the shift of a water proton along a H-bridge from the ligand H_2O to N_ϵ of Im, His E7 in such a way that a low-spin complex similar to $MetHbOH^-$ is supposed to be formed. Both reactions should be influenced differently by structural changes in the heme region, as explained in more detail in 4.1. We must therefore discuss carefully, which reaction is responsible for the ms-relaxation.

First, the large reaction enthalpy measured here, and in ethyleneglycol–water mixtures at low temperatures in [4], corresponds to the ΔH-values given in [12] for the binding of Im to MetHb. This argues for model I. Two further arguments are: (i) In [13] reactions are investigated which lead to a 1:1 complex of Im with ferriprotoporphyrine IX, substituted by ethylenediamine in aqueous solution. For the reverse reaction step, where Im dissociates and is replaced by H_2O, at pH 5, 25°C as velocity constant it was found $k_{off,Im} = 2.2 \times 10^3\ s^{-1}$ or $2 \times 10^4\ s^{-1}$ resp. depending on H_2O or OH^- being ligand *trans* to the Im. By way of comparison, with MetHb, pH 6, we calculate at 25°C $k_{off} = 1.1 \times 10^3\ s^{-1}$. This order-of-magnitude agreement between $k_{off,Im}$ and k_{off} we consider as an argument that the k_{off} determined with MetHb corresponds to the dissociation of Im, His E7 from the heme iron in the course of the replacement reaction, model I. If model II (proton displacement) would be the basis of the ms-relaxation, one would expect essentially higher relaxation rates. (ii) Fig.1 shows the kinetic difference spectrum of the ms-relaxation. It is in good agreement with the stationary difference spectrum $MetHbH_2O$-MetHbIm, but not with the one of $MetHbH_2O$-$MetHbOH^-$. This is a further argument in favour of model I and against model II.

These arguments lead us to the interpretation of

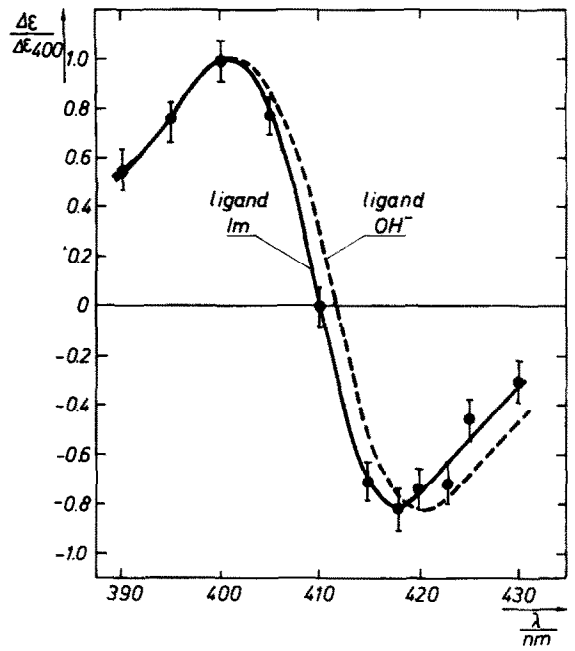

Fig.1. Difference spectra, all normalized to 1 at 400 nm. ⧫, Kinetic difference spectrum $\Delta\epsilon$ (λ) of MetHb, pH 6, ΔT = 5°C, T = 7°C. $\Delta\epsilon$ (400) = 0.217/mM/cm. ——, Stationary difference spectrum $\Delta\epsilon$ (λ) between $MetHbH_2O$ and $MetHbH_2O$ + Im. [MetHb] = 10^{-5} M, pH 6, T = 7°C. The curve is an average of [Im] = 0.01 and 0.02 M with zeros at (410.2 ± 0.6) and (410.5 ± 0.6) nm, resp. $\Delta\epsilon$ (400) = 28.1/mM/cm for [Im] = 0.02 M. ——, Stationary difference spectrum $\Delta\epsilon$ (λ) between $MetHbH_2O$, pH 6 and pH > 6. [MetHb] = 10^{-5} M, T = 7°C. The curve is an average of pH 7.7 and pH 8.0 with zeros at (412.1 ± 0.3) and (412.4 ± 0.3) nm, resp. $\Delta\epsilon$ (400) = 23.6/mM/cm at pH 8. The stationary spectra were successively measured (Beckman acta V) with the same optical cuvette, digitally stored (Fabritec 1072), and subtracted. The difference spectra were then folded computationally to account for the different optical bandwidths $\Delta\lambda$ (0.2 nm stationary, 6 nm kinetic). Thus all spectra correspond to $\Delta\lambda$ = 6 nm.

Eqn. 1 that in the state A we have H_2O as the ligand of the heme iron, in the state B it is Im,His E7, the H_2O being displaced. The relaxation rate observed here is determined by the dissociation step of that reaction.

3.3. *Calculations on the position(s) of N_ϵ, Im,His E7*

With atomic coordinates of [14] we calculated the distance r between N_ϵ of Im,His E7 and Fe for MetHb on the simplifying assumptions that (i) position and orientation of C_α (His E7) and Fe remain fixed, and (ii) both C-C bonds of His E7 are free to rotate without sterical hindrance. The ranges for r calculated herewith are r_α = 4.33–8.58 Å for the α-chain and r_β = 4.20–8.46 Å for the β-chain. By way of comparison, the X-ray data for the distance r in the crystal are $r_{\alpha,X}$ = 4.36 Å, $r_{\beta,X}$ = 4.21 Å. Thus it turns out that in the crystal the shortest possible distances N_ϵ-Fe are realized. This holds for both chains.

4. Conclusions

4.1. *Thermal fluctuations of tertiary structure in the heme region*

If we consider the X-ray coordinates of C_α (His E7) and Fe in a first approximation as the time average of the coordinates in aqueous solution, we have to postulate large thermal fluctuations in this region: direct bonding Im-Fe (model I) is then possible only if the distance r between N_ϵ and Fe is lowered by nearly 2 Å. This cannot be realized (see 3.3) by means of the two rotational degrees of freedom in His E7 alone, as r takes the smallest possible value in the crystal already. Rather E-helix and heme have to carry out considerable thermal fluctuations relative to each other in order to reduce r by about 2 Å. This may also explain the small value of $k_{on} \sim K/\tau \sim 1\ s^{-1}$. The inferences made in 4.1 are based on the result that model I and not model II is responsible for the ms-relaxation. In the framework of the (excluded) model II the situation would be quite different: there the important H-bridge N_ϵ-H_2O along which the proton is assumed to be shifted can be even closed and opened without noticeable changes in the distance r, therefore we could not directly infer from the ms-relaxation the existence of large thermal fluctuations of the tertiary structure.

4.2. *Limitations of energy refinement calculations (ERC) by fluctuations of macromolecular structure: an example*

For the equilibrium constant K (Eqn. 1) we obtained

$$K_{20°C} = [B]/[A] = \left[\exp\left(-\frac{\Delta H}{RT}\right)\right]\left[\exp\left(\frac{T\Delta S}{RT}\right)\right] \sim \left[3 \times 10^{11}\right]\left[4 \times 10^{-15}\right]$$

The energetically strongly favoured state B (His E7 bonded) is thus – in agreement with X-ray data – only little occupied here due to the large entropy

term ΔS. It is shown in [15] that by ERC detailed statements on structure and function of Hb are possible. This is done by minimizing the total potential energy while the entropy remains unconsidered. In our case such a calculation would yield the paradoxical result that practically only state B, the hemichrome does exist. This is due to the fact that ERC considers only energy, whereas at room temperature fluctuations result in a large entropy term $T\Delta S$ which does even overcompensate the strongly negative energy term ΔH. So entropy rather than energy contributions may in certain cases be the determining factor for protein structure and function.

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References

[1] McCammon, J. A., Gelin, B. R. and Karplus, M. (1978) Nature 267, 585–590.
[2] Beetlestone, J. G. and Irvine, D. H. (1968) J. Chem. Soc. (A), 1340–1346.
[3] Rein, H., Ristau, O. and Ruckpaul, K. (1975) Biochim. Biophys. Acta 393, 373–378.
[4] Dreyer, U. and Ilgenfritz, G. (1979) Biochem. Biophys. Res. Commun. 87, 1011–1017.
[5] Eigen, M. and DeMaeyer, L. (1963) in: Technique of Organic Chemistry (Friess, S. L., Lewis, E. S. and Weissberger, A. eds) vol. VIII, part II, pp. 895–1051, Interscience, New York.
[6] Bracht, A., Eufinger, B. R., Redhardt, A. and Schlitter, J. (1979) Biochem. Biophys. Res. Commun. 86, 585–593.
[7] Perutz, M. F. (1979) Annu. Rev. Biochem. 48, 327–386.
[8] Benesch, R. E., Benesch, R., Renthal, R. D. and Maeda, N. (1972) Biochemistry 11, 3576–3582.
[9] Bernasconi, C. F. (1976) Relaxation Kinetics, Academic Press, New York.
[10] George, P., Beetlestone, J. and Griffith, J. S. (1964) Rev. Mod. Phys. 36, 441–458.
[11] Schwartz, A. M. and Schimmel, P. R. (1974) J. Mol. Biol. 89, 505–510.
[12] Beetlestone, J. G., Epega, A. A. and Irvine, D. H. (1968) J. Chem. Soc. (A) 1346–1351.
[13] Kolski, G. B. and Plane, R. A. (1972) J. Am. Chem. Soc. 94, 3740–3744.
[14] Ladner, R. C., Heidner, E. G. and Perutz, M. F. (1977) J. Mol. Biol. 114, 385–414.
[15] Gelin, B. R. and Karplus, M. (1977) Proc. Natl. Acad. Sci. USA 74, 801–805.