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Subunit iron spin heterogeneity in human aquomethemoglobin A

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On the basis of a reaction scheme in which the ligand binding steps are preceded by fast iron spin transitions (Okonjo, K.O. (1980) Eur. J. Biochem. 105, 329–334; Iwuoha, E.I. and Okonjo, K.O. (1985) Biochim. Biophys. Acta 829, 327–334), the spin equilibrium constants of methemoglobin subunits are calculated from kinetic and equilibrium binding parameters with azide ion as ligand. The results demonstrate the existence of thermodynamic spin heterogeneity within the tetramer.

Introduction

The role of the iron spin state transition in the structural transformations accompanying the normal physiological function of hemoglobin is not clear and has been the subject of controversy [1–6]. This controversy arose from Perutz's suggestion that the T → R allosteric transition in ferrohemoglobin is triggered by a spin change of the iron atoms from high to low spin [7]. In two recent reports [8,9] we demonstrated that the iron spin transition plays an important role in the binding of ligands to aquomethemoglobin. Kinetic and relaxation amplitude data were satisfactorily analysed in terms of a scheme (Scheme I) in which the ligand binding steps of the α and β subunits within the tetramer are preceded by a fast iron spin transition [8,9]:

$$\alpha + L \underset{k^{lh}}{\overset{k^{hl}}{\rightleftarrows}} \alpha^* + L \underset{k^{\alpha}_{-L}}{\overset{k^{\alpha}_{L}}{\rightleftarrows}} \alpha^* L$$

$$\beta + L \underset{k^{lh}}{\overset{k^{hl}}{\rightleftarrows}} \beta^* + L \underset{k^{\beta}_{-L}}{\overset{k^{\beta}_{L}}{\rightleftarrows}} \beta^* L \qquad \text{(Scheme I)}$$

In this scheme, i and i^* are the high-spin and low-spin forms of subunit i, respectively; k^{hl} (k^{lh}) is the rate constant of the high to low spin (low to high spin) transition; k^i_L (k^i_{-L}) is the association (dissociation) rate constant for the binding of ligand L. In the presence of excess ligand the reciprocal relaxation time for ligand binding to subunit i is given [8,9] by:

$$\tau_i^{-1} = k^i_L C_L + k^i_{-L}(1 + k^{lh}/k^{hl}) \qquad (1)$$

The underlying assumption in these analyses [8,9] is that K_{spin}, that is, k^{lh}/k^{hl} of Eqn. 1, is the same for the α and β subunits within the methemoglobin tetramer. This assumption is based on the observation that the kinetics of the iron spin transition are characterized by only one relaxation phase [10]. Moreover, the methods used for the determination of magnetic susceptibilities do not allow for a distinction between the spin contributions of the α and β subunits within the methemoglobin tetramer.

The ligands employed in our previous studies [8,9] are formate and thiocyanate ions. They belong to that class of ligands which bind to methemoglobin with kinetic heterogeneity but with thermodynamic homogeneity [11]. A test of the

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assumption of iron spin homogeneity in methemoglobin requires a ligand that binds to methemoglobin with kinetic and thermodynamic heterogeneity. Azide ion belongs to this class of ligands [11].

From Eqn. 1 a plot of τ_i^{-1} against the total ligand concentration, C_L, should give a straight line of slope k_L^i and intercept k_D^i where

$$k_D^i = k_{-L}^i(1 + k^{lh}/k^{hl}) \quad (2)$$

Hence

$$k_L^i/k_{-L}^i = k_L^i(1 + k^{lh}/k^{hl})/k_D^i \quad (3)$$

Let K_{equ}^i be the subunit complex formation constant, as determined by equilibrium titration. We show below that Scheme I is an adequate description of the binding of azide ion to methemoglobin. It is therefore reasonable to assume that Scheme I is also an adequate description of the binding of azide ion to each subunit within the methemoglobin molecule. Therefore one may write

$$k_L^i/k_{-L}^i = K_{equ}^i \quad (4)$$

Assuming subunit spin heterogeneity one obtains from Eqns. 3 and 4

$$1 + k_i^{lh}/k_i^{hl} = k_D^i K_{equ}^i/k_L^i \quad (5)$$

It should thus be possible to calculate the spin equilibrium constant for each methemoglobin subunit i with the aid of Eqn. 5, provided the kinetic constants (k_L^i, k_D^i) and the subunit complex formation constants (K_{equ}^i) are known.

We have carried out a kinetic study of the reaction of azide ion with methemoglobin. Using the subunit equilibrium binding parameters determined by Beetlestone [12], together with our kinetic parameters, we demonstrate that the β subunit spin equilibrium constant, K_{spin}^{β}, is almost 6-fold greater than that of the α subunit. Thus methemoglobin subunits display spin heterogeneity within the tetramer.

Materials and Methods

The kinetics were studied under pseudo-first-order conditions with a Unicam SP 30UV spectrophotometer. The experimental conditions were identical to those of the equilibrium binding studies [12]. The cell compartment was thermostatted with a Lauda TUK 30 Table Cryostat. Solutions of methemoglobin (2 μM heme) were prepared in phosphate buffer pH 6.0, ionic strength 0.05 M. These solutions were allowed to temperature-equilibrate in the Lauda thermostat at 20°C. For each solution a 3 ml aliquot was pipetted into a spectrophotometric cell and the cell was thereafter placed in the cell compartment of the spectrophotometer. After allowing for temperature equilibration the absorbance of the solution in the cell was recorded. A few microlitres of an azide solution were then added with a microstirrer. The final azide concentration in the cuvette ranged between 20 and 50 μM. The absorbance of the mixture was recorded at intervals of time, at 405 nm. Reactions were followed for about two half-lives before a few crystals of NaN_3 were added to obtain the infinity reading. The kinetic data were analysed on an IBM 370 computer of the University of Ibadan by a Fourier method of analysis of exponential curves[13,14].

Results and Discussion

Fig. 1 shows the results of a typical kinetic run. The theoretical curve is based on a two-exponential fit of the data:

$$\Delta E_t = \Delta E_\alpha^0 \exp(-k_\alpha t) + \Delta E_\beta^0 \exp(-k_\beta t) \quad (6)$$

Two-phase kinetics have been observed before for azide binding to methemoglobin, and the phases were assigned to the binding of azide ion to α (slow phase) and β (fast phase) subunits [15]. In Eqn. 6 ΔE_t is the absorbance change at time t and ΔE_i^0 is the total absorbance change of subunit i when it is saturated with azide. For the run presented in Fig. 1 $\Delta E_\alpha^0 = 5.95 \cdot 10^{-2}$ and $\Delta E_\beta^0 = 11.39 \cdot 10^{-2}$. Thus the total absorbance change due to the α subunits is about 34% of the total. This result is similar to that in Ref. 15 where it was found that the two kinetic components did not contribute equally to the absorbance changes, the contributions depending on wavelength, ionic strength, pH and type of buffer [16].

Fig. 2 illustrates the dependence of the

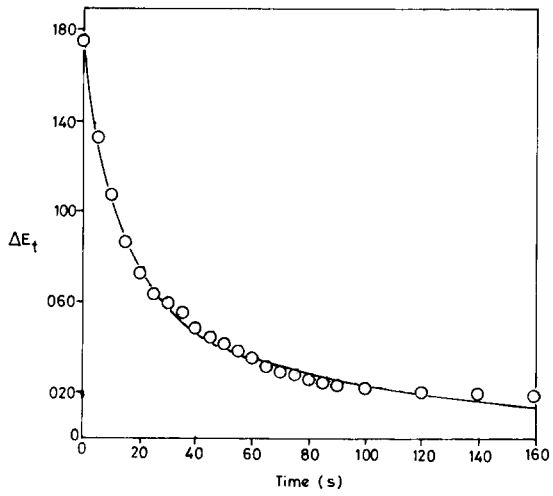

Fig. 1. Time course of the reaction of azide ion with aquomethemoglobin A. The theoretical line was calculated with the equation: $\Delta E_t = \Delta E_\beta^0 e^{-k_\beta t} + \Delta E_\alpha^0 e^{-k_\alpha t}$. The fitting parameters are $\Delta E_\alpha^0 = 5.95 \cdot 10^{-2}$; $\Delta E_\beta^0 = 11.39 \cdot 10^{-2}$; $k_\alpha = 9.03 \cdot 10^{-3}\ S^{-1}$; $k_\beta = 75.5 \cdot 10^{-3}\ S^{-1}$. Conditions: phosphate buffer pH 6.0; ionic strength 0.05 M (added salt, NaCl); 20 °C. The working wavelength was 405 nm. Methemoglobin concentration, 2 μM heme; azide concentration, 30 μM.

pseudo-first-order rate constants k_i ($i = \alpha, \beta$) on azide concentration. Each point is an average from at least three separate kinetic runs. Linear plots

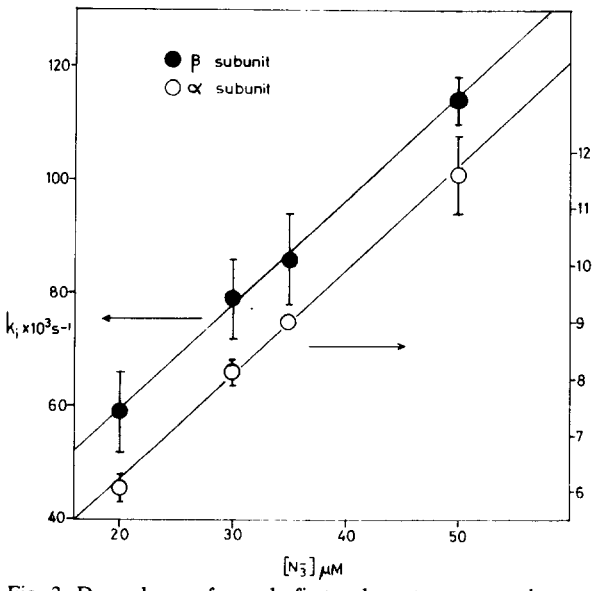

Fig. 2. Dependence of pseudo-first-order rate constant k_i on azide concentration. Conditions as in Fig. 1. Error bars indicate standard errors from at least three kinetic runs. Left-hand ordinate: fast (or β) phase; right-hand ordinate: slow (or α) phase.

TABLE I

KINETIC AND EQUILIBRIUM PARAMETERS FOR THE REACTION OF AZIDE ION WITH METHEMOGLOBIN SUBUNITS WITHIN THE TETRAMER

Compare with Fig. 2, Scheme I and Eqn. 7. k_D^i are the intercepts of the plots in Fig. 2 and are related to k_{-L}^i of Scheme I by Eqn. 2. Values of K_{equ}^i are from Ref. 12.

Parameters	α subunit	β subunit
k_L ($M^{-1} \cdot S^{-1}$)	183.1	1836.7
$10^3\ k_D$ (S^{-1})	2.5	22.7
$10^{-5}\ K_{equ}$ (M^{-1})	2.29	10.5
K_{spin}	2.15	11.94

are obtained for each subunit according to the equation

$$k_i = k_L^i C_L + k_{-L}^i \left(1 + k_i^{lh}/k_i^{hl}\right) \quad (7)$$

as expected from Scheme I. An underlying assumption in Eqn. 7 is that the two kinetic phases are not coupled. This assumption is justified under pseudo-first-order conditions [8,9]. Furthermore, an examination of the kinetic rate constants (Table I) shows that those of the β subunits are an order of magnitude higher than those of the α subunits. This further justifies the assumption of uncoupled kinetic steps. k_L^i are the least-squares slopes and k_D^i are the least-squares intercepts of Fig. 2. Eqn. 2 relates k_D^i to k_{-L}^i of Scheme I. On the assumption of spin heterogeneity, k_i^{lh} (k_i^{hl}) replaces k^{lh} (k^{hl}) in Eqn. 2.

The spin equilibrium constant, K_{spin}^i, that is, k^{lh}/k^{hl}, for each subunit i was calculated from Eqn. 5 using the kinetic parameters k_L^i and k_D^i of Fig. 2 and the subunit azide binding constants, K_{equ}^i, determined [12] by equilibrium titration. The kinetic and equilibrium binding constants are collected in Table I, which also shows the calculated values of K_{spin}^i. It is seen that K_{spin}^β, with a value of 11.94, is strikingly different from K_{spin}^α, which is 2.15. This result demonstrates that the methemoglobin subunits exhibit different thermodynamic spin characteristics, as opposed to the homogeneous kinetic characteristics reported before [10]. From our data we calculate that the α subunits are 67% high-spin whereas the β subunits are 92% high-spin.

In view of the spin heterogeneity reported here

for the subunits of methemoglobin A, it is necessary to justify, for azide ion as ligand, our method of analysis which is based on an assumption of subunit (thermodynamic) spin homogeneity [8,9]. Moreover, the ligands for which Scheme I has been found to be adequate (formate and thiocyanate ions) are high-spin ligands, whereas azide ion is a low-spin ligand. We have therefore used the same method of analysis as before [8,9] to test the applicability of Scheme I to the binding of a low-spin ligand, in this case azide ion. As before [8,9], the value of k^{lh}/k^{hl} used in the analysis was derived from Anusiem's magnetic susceptibility data [17]. At 20°C this value is 5.45. According to our method of analysis the following relationship should hold true if Scheme I is applicable, with azide ion as ligand:

$$\left(k_L^{\alpha} k_L^{\beta} / k_{-L}^{\alpha} k_{-L}^{\beta}\right)^{1/2} = \left(K_{equ}^{\alpha} K_{equ}^{\beta}\right)^{1/2}$$

The values obtained are $4.94 \cdot 10^5$ M^{-1} for the expression on the left, and $4.90 \cdot 10^5$ M^{-1} for the expression on the right of the above equation, respectively. Thus Scheme I is applicable to the binding of azide ion. We conclude that Scheme I provides an adequate description of the binding of high-spin as well as of low-spin ligands to methemoglobin, and that the use of average spin equilibrium constants from susceptibility measurements on the methemoglobin molecule is justified. Since subunit spin equilibrium constants are not accessible to direct measurement, average spin equilibrium constants may be used in their stead for the methemoglobin tetramer.

Subunit heterogeneity in methemoglobin has been reported before on the kinetics [15] and thermodynamics [12,18] of ligand binding, on the spectra of the subunits within the tetramer [15,16], and on the kinetics of reduction of met subunits and oxidation of deoxy subunits [16]. Different spin characteristics have been reported for the isolated methemoglobin chains [19]. Subunit spin heterogeneity is suggested by a comparison of the infrared data on the spin characteristics of the azide complex of methemoglobin A and M Milwaukee [20]. Our finding of subunit spin heterogeneity in aquomethemoglobin (Table I) is one more example of subunit heterogeneity in hemoglobin.

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