Volume 81, number 1 FEBS LETTERS September 1977

PULSE-RADIOLYTIC STUDIES ON THE SPIN-STATE TRANSITIONS IN AQUOMETHEMOGLOBIN AFTER REDUCTION OF A SINGLE HEME GROUP

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Received 30 May 1977
Revised version received 2 July 1977

1. Introduction

We have previously observed a transient state (half-time ∿ 15 μs) in aquomethemoglobin with an absorption maximum near 420 nm after rapid reduction of a single ferric heme group by hydrated electrons [1]. This observation has been confirmed [2,3]. Moreover, this microsecond process disappeared in the presence of Ins-P_6 [1]. On binding this organic phosphate, metHb changes its quaternary conformation from the *R*- to the *T*-state [4]. Therefore, we concluded that in the absence of an allosteric effector the reduction reaction of one heme group in metHb by hydrated electrons was followed by a quaternary conformational change ($R \rightarrow T$ transition).

Recently, a number of studies on the kinetics of the binding of carbon monoxide [5] and oxygen [3,6] to a reduced single heme group of metHb provided on-rate constants characteristic for the *R*-state. These pulse-radiolytic experiments indicated that at low reduction degrees no change in quaternary conformation takes place. In an attempt to elucidate this apparent discrepancy, we have investigated the spectral properties of the transient state.

The data show that the absorption spectrum of the transient state has the characteristics of a ferrous low spin-state. The subsequent transition of the ferrous low spin to the stable ferrous high spin-state proceeds with a half-time that depends on the solvent condition. On the basis of these results we have postulated a two-step reduction mechanism for aquomethemoglobin.

2. Materials and methods

Fresh human hemoglobin was isolated by Drabkin's methods [7] and stripped of organic phosphates by passing the solutions through a mixed-bed, ion-exchange column (Amberlite, IRA 400 and IR 120). Methemoglobin was obtained by adding a 1.5-fold excess of potassium ferricyanide to oxyhemoglobin solutions. The excess ferricyanide anions were removed on a Sephadex G-25 column, and then the methemoglobin solutions were passed through a mixed-bed, ion-exchange column.

The various metHb preparations were diluted in a 3 mM phosphate buffer (pH 7.2) containing methanol. All solutions were de-aerated before irradiation with 550 ns pulses of about 1 krad from a 2 MV Van de Graaff electron accelerator. During the radiolysis of aqueous solutions hydrated electrons ($\cdot e_{aq}^-$) are produced together with hydroxyl (·OH) and hydrogen (·H) radicals which both are scavenged by methanol by means of hydrogen abstraction [8]. The reduction degree of metHb was kept low (< 0.03) so that the

Abbreviations: $\cdot e_{aq}^-$, hydrated electrons; ·H, hydrogen radical; ·OH, hydroxyl radical; HB^{3+}, methemoglobin with one reduced heme group; Ins-P_6, inositol hexaphosphate concentration of hemoglobin molecules with two reduced heme groups could be neglected.

After the electron-pulse, changes in the transmission of the sample were measured with a light detector (4840, RCA-photomultiplier) and recorded by a Digital Processing Oscilloscope (DPO-system, Tektronix). The absorbance changes in the Soret and visible wavelength regions corresponding to the rapid reduction and decay of the transient state were digitized and then analyzed according to the eigenfunction expansion method of Provencher [9,10]. For each experiment the concentration of the reduced heme groups was calculated from the total change in absorbance observed after 500 µs at 435 nm. These wavelengths are isosbestic points for the quaternary *R*- and *T*-states of deoxyhemoglobin [11]. All experiments were performed at room temperature.

3. Results and discussion

Two examples of the digitized time-recording of the absorbance changes at 424 nm and 560 nm are shown in fig.1. At both wavelengths the change in absorbance of the reduction and relaxation processes have opposite signs while the relative contribution of the amplitudes is optimal. The (sub)-microsecond absorbance change represents the actual reduction process. This can be demonstrated by following the disappearance of the absorbance of the hydrated electrons at 650 nm [1,2]. The decay of the transient state observed after the reduction of the ferric heme group is found to be exponential. The half-time of this process is independent of the heme and hydrated electron concentrations.

The kinetic spectrum of the transient and final states are shown in fig.2. The former is typical for a low spin ferrous heme. The large α-band at 559 nm, the smaller β-band at 525 nm and the Soret- or γ-band at 423 nm resemble a hemochrome spectrum [12]. The kinetic spectrum of the final state differs from that of Hb in the *T*-state but corresponds to that of Hb in the *R*-state. The reduced heme group of this state is unliganded and has been shown to react with carbon monoxide [5] and oxygen [3,6].

The formation of a hemochrome indicates that large structural changes in the heme environment accompany the electron transfer process. Under our experimental conditions the reduction reaction leading to the ferrous low spin-state is virtually complete within the duration of the electron pulse. We have estimated an upper limit for the relaxation-time of 400 ns for the rearrangements of the protein structure in the vicinity of the heme group accompanying the reduction process. Tertiary conformational changes in the structure of hemoglobin on the submicrosecond time-scale have been reported by Alpert et al. [13] from flash photolysis studies. It is still difficult to relate the transient state observed after ligand removal by flash photolysis of carboxyhemoglobin to the transient state reported here.

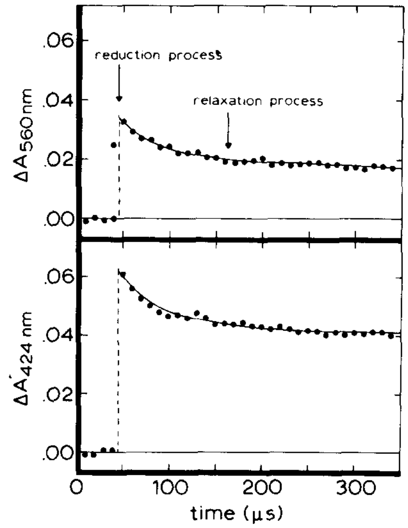

Fig.1. Transient signals of the reduction and relaxation process recorded after pulse radiolysis of aqueous methanol solutions containing metHb. The absorbance differences at 560 nm (upper trace) and 424 nm (lower trace) initially show a step in the absorbance due to the reaction of the ferric heme group with the hydrated electrons. Subsequently, a slower decay is observed corresponding to a low spin- to a high spin-state transition of the ferrous heme group. Experimental conditions: Heme concentrations respectively 109 µM and 8.6 µM on heme basis; 3 mM phosphate (pH 7.2); 1 M methanol; reduction degree 0.03.

Recently Blumenfeld et al. [14] observed an absorption spectrum after γ-irradiation of methemoglobin in water–ethylene glycol solutions at liquid-nitrogen temperature very similar to that of the low spin hemochrome spectrum reported here. The low-temperature spectrum is approx. 2 nm blue-shifted

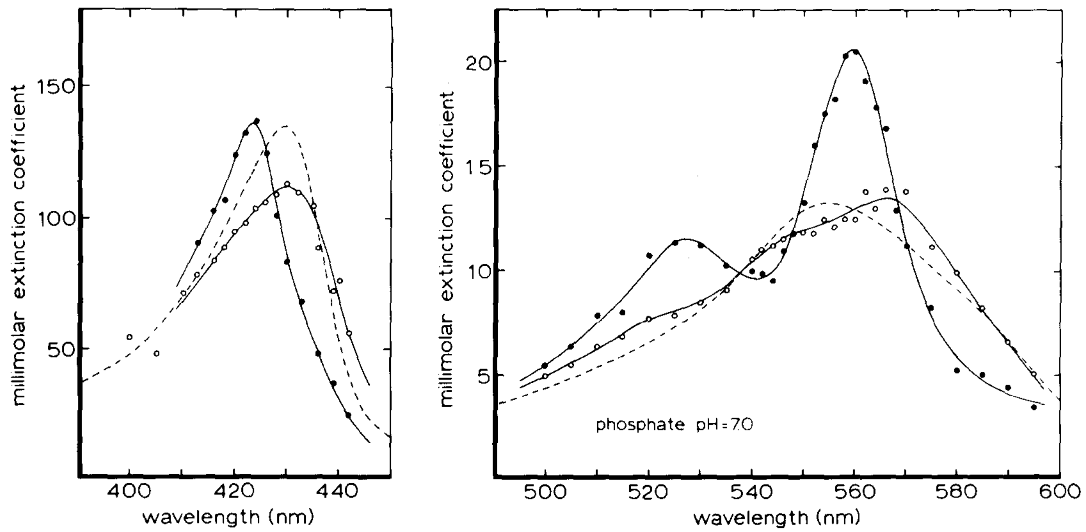

Fig.2. The kinetic absorption spectra in the Soret and visible wavelength region obtained after single reduction of the heme groups in aquomethemoglobin by hydrated electrons. The absorption spectrum observed immediately after the disappearance of the hydrated electron (●) corresponds to the actual reduction of the ferric heme group to a characteristic low spin ferrous form of hemoglobin. The relaxation process produces a final absorption spectrum (○) reminiscent of a high spin ferrous form of hemoglobin. The latter spectrum deviates from that of deoxyhemoglobin in the quaternary *T*-state (dashed line). The calibration of millimolar extinction coefficients of the kinetic absorption spectra is performed at 435 nm and 540 nm, respectively, (isosbestic wavelengths of the quaternary *R*- and *T*-state). For experimental conditions see fig.1.

with respect to the spectrum shown in fig.2, a shift ascribable to the temperature difference. We have concluded therefore that the ferrous low spin-state observed in our experiments corresponds to that reported by Blumenfeld et al. [14], who have proposed several hypotheses to explain it. Our kinetic experiments confirm one of these, namely that an endogeneous nitrogen-base is involved as a sixth ligand during the electron transfer process. Most probably the distal-histidine acts as the primary electron acceptor of the subunit. Subsequently, the electron transfers from the distal-histidine to one of the *d*-orbitals of the iron atom leading to a (unstable) ferrous low spin-state.

The transition of the low spin transient to the final high spin-state of the ferrous heme group possesses a half-time dependent on the methanol concentration as shown in the double logarithmic plot of fig.3. We suggest that this effect is due to a coupling of the dynamic polarization of the solvent and the vibronic modes of the heme groups [15]. Probably an important fact in connection with this coupling is the presence of water molecules in the heme pocket leading to a relatively polar environment of the heme group [16,17]. These considerations suggest the following reduction and relaxation scheme:

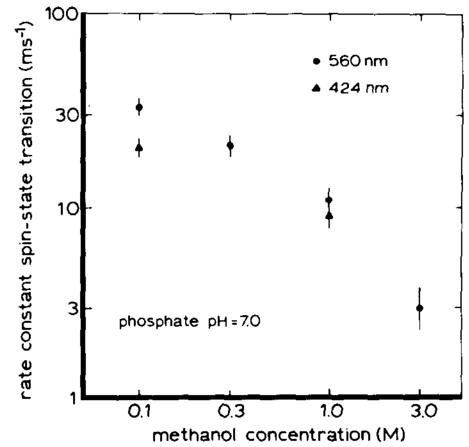

Fig.3. The effect of methanol on the first order rate constant of the spin-state transition observed in the Soret and visible region. Experimental conditions: 50 μM metHb on heme basis; 3 mM phosphate (pH 7.2); reduction degree 0.02.

Scheme

$$\left.\begin{array}{l}[\mathrm{Hb}(\text{heme } d^5_{1/2})\mathrm{OH}^- \ldots \mathrm{.His}] \\ [\mathrm{Hb}(\text{heme } d^5_{5/2})\,\mathrm{OH}_2 \ldots \mathrm{.His}]\end{array}\right\} + \cdot e^-_{aq} \xrightarrow{k_{red}} [\mathrm{Hb}(\text{heme } d^6_0)\mathrm{His} \ldots \mathrm{.H_2O}] \xrightarrow{k_{rel}} [\mathrm{Hb}(\text{heme } d^6_2)\mathrm{unl} \quad \mathrm{His} \ldots \mathrm{.H_2O}]$$

in which the notation of Peisach et al. [18] is used. Here k_{red} represents the second order rate constant for the reduction of a single heme group, whereas k_{rel} is the first order rate constant for the relaxation of the transient state. For k_{red} we have measured a value of 16 mM^{-1} μs^{-1}, at pH 7.2 [1].

The final absorption spectrum observed after the spin-state transition differed from that of normal ferrous high spin deoxy-hemoglobin at all heme concentrations measured, but corresponded to the spectrum of the deoxy *R*-state or rapidly reacting forms of Hb first observed by Gibson [19]. This result is in accordance with the observations that ligand binding to Hb^{3+} proceeds with on-rate constants characteristic for the *R*-state [3,5,6]. The *R*-type Hb-spectrum is also found in the Soret-region, where under our experimental conditions metHb is largely dissociated into dimers [20]. This observation is in accordance with the fact that Hb-dimers also show this type of spectrum [21].

Finally we want to emphasize that the effect of methanol on the stability of the reduced subunit together with its hemochrome spectrum indicate that the water molecule present in aquomethemoglobin is released before the formation of the hemochromogen. Further investigations on the spin-state transition in partially reduced metHb are in progress.

Acknowledgement

The authors thank the Netherlands Organization for the Advancement of Pure Research (ZWO) for providing financial assistance and especially for supplying the DPO-system.

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