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CHAIN HETEROGENEITY AS DEMONSTRATED BY
THE FERRICYANIDE OXIDATION OF THE ONE-SITE REDUCED BOVINE METHEMOGLOBIN

PROGRESS REPORT

Yael A. Ilan and Gidon Czapski

Department of Physical Chemistry
The Hebrew University of Jerusalem, Israel

Work carried out during the period:
December 1st 1977 to November 30th 1978

Prepared for the U.S. Energy Research & Development Administration
Under Contract No. EY-76-C-02-3221

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Summary. With the pulse radiolysis technique a single chain of tetrameric bovine methemoglobin ($\alpha_2^{\text{III}}\beta_2^{\text{III}}$) is reduced producing a mixture of valence hybrids consisting of $\alpha^{\text{III}}\alpha^{\text{II}}\beta_2^{\text{III}}$ and $\alpha_2^{\text{III}}\beta^{\text{III}}\beta^{\text{II}}$. The oxidation of these partially reduced hemoglobins with ferricyanide was studied. It was found that the rate of oxidation of one of them proceeds 6-11 times faster than the other. It is suggested that the fast reacting valence hybride contains reduced alpha chains.

Introduction

The oxidation of tetrameric hemoglobin with ferricyanide has been used for years for the preparation of methemoglobin. The reaction proceeds by a single electron transfer from heme iron to ferricyanide producing oxidized ferric heme as a product. Although the overall reaction has been studied in great detail (1), there is less information available concerning the oxidation of individual chains in the tetramer and the kinetics of these processes.

In this present communication, we describe the oxidation of partially reduced tetrameric methemoglobins containing only a single reduced chain. These valence hybrid hemoglobins were prepared using

pulse radiolysis (2), with this technique, hydrated electrons reduce methemoglobin to produce two mixed state hybrids (3,4), in equal concentration.

As hydrated electrons are highly unselective as reducing agents, we can assume that α and β chains are reduced equivalently. The reduction reaction is diffusion controlled (4) and provided that the total number of hemes reduced in the preparation of ferric tetramers is small, then under these conditions only a single chain is reduced in any tetramer. From these studies, we find that the rate of oxidation of partially reduced valence hybrids is biphasic. As only single chain in any tetramer is oxidized it is suggested that the biphasicity is due to the presence of two different valence hybrids, one containing a single reduced alpha chain and the other a single reduced beta chain.

Experimental

Bovine hemoglobin and horse heart metmyoglobin were purchased from Sigma. All chemicals were of the highest analytical grade available and were used without any further purification. Solutions were prepared in triply distilled water immediately before irradiation.

Details of the pulse radiolysis technique were previously described (4). Pulse duration was 0.5-1.5 usec (200mA, 5MeV) and the e_{aq}^- concentration was $5-10 \times 10^{-6}$ M. A spectrosil cell with optical path of 1 cm was used. Appropriate light filters were used to minimize photochemical and scattered light effects.

All solutions contained 0.1M butanol in order to scavenge $\cdot\text{OH}$ radicals and leave only e_{aq}^- as the reactive species (2). The pH was adjusted with phosphate buffer at pH 6.3 and 7.0 and with borax buffer at pH 9.2. The ionic strength was 0.02. All solutions were saturated with Ar. Ferricyanide concentrations were $2-5 \times 10^{-4}\text{M}$ at pH 6.3, $0.5-50 \times 10^{-4}\text{M}$ at pH 7.0 and $1-2 \times 10^{-4}\text{M}$ at pH 9.2. Methemoglobin concentrations were $8-16 \times 10^{-5}\text{M}$ in heme. The concentration of e_{aq}^- was measured spectrophotometrically at 578 nm where $\epsilon_{e-\text{aq}} = 10,600 \text{ M}^{-1} \text{ cm}^{-1}$ (2).

Results and Discussion

We studied the oxidation of one-site reduced methemoglobin as a function of ferricyanide concentration in the pH range 6.3-9.2. The rate constants were measured both at 560 and 440 nm and at these wavelengths the absorbance decayed in two pseudo first order processes. Both rate constants, k_2 and k_3 (Table 1) were proportional to ferricyanide concentration as shown in Fig. 1. Addition of 10^{-3}M ferrocyanide had no effect on the reaction. By extrapolation to zero time for both of these decays, it was found that the optical change was derived from two species each absorbing equally.

Since the level of reduced hemes in the sample subjected to pulse radiolysis is very small and the hydrated electron has equal probability in the reactivity with either alpha or beta chains in a hemoglobin tetramer (Eq. 1), then we may write the oxidation process as two different reactions.

and

Here the oxidation with ferricyanide occurs with two different valence hybrids, one which contains a single reduced alpha chain (Eq. 2) and the other, a single reduced beta chain (Eq. 3).

Table I summarizes the kinetic results of the oxidation process as a function of pH. As can be seen, both rate constants k_2 and k_3 are pH dependent and are attributed to the oxidation of the two species given in equations 2 and 3. When isolated, α and β chains have different redox potentials ($E_{m7}^{\beta} = 0.113V$), $E_{m7}^{\alpha} = 0.052V$ (5)) and it seems likely that within the tetramer, there should be differences as well (6). Provided that the kinetics of the reaction between ferricyanide and reduced heme is directly correlated with the redox potential, then it is suggested that k_2 is ascribed to the oxidation of ferrous alpha chains within the hemoglobin tetramer (Eq. 2) and k_3 to the oxidation of ferrous beta chains within the hemoglobin tetramer (Eq. 3).

At pH 9.2, k_3 is an order of magnitude smaller than k_2 (Table I). The slower rate k_3 , is comparable to the value ($0.8 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$) obtained from a study of the oxidation of fully reduced methemoglobin A ($\alpha_2^{\text{II}} \beta_2^{\text{II}}$) using the stopped flow technique (1). At pH 6.3 the ratio of k_2 to k_3 is somewhat smaller. However, the value of k_3 is faster than that obtained using the stopped flow procedure ($7 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$). The reasons for the small discrepancy may be due to species differences or to other phenomena not clear to us at this time.

In the same way that methemoglobin can be reduced by the pulse radiolysis technique, metmyoglobin can be reduced as well.

The reduced myoglobin can now be reacted with ferricyanide in a fashion comparable to the oxidation of single site reduced methemoglobin.

The rate of oxidation we obtained at pH 7 ($2.9 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$) is within the range obtained for this same oxidation ($1.8 - 5.0 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$) (1) using a variety of techniques (1).

In conclusion, we have demonstrated functional heterogeneity in two mixed state tetrameric hemoglobins in which a single chain is reduced each, for the oxidative reaction with ferricyanide. Although this finding is suggested from analogous studies where fully reduced tetrameric hemoglobin is reacted with ferricyanide (7), our study is the first to demonstrate heterogeneity in oxidation properties of the final step of the overall reaction.

This conclusion seems reasonable since in the autoxidation of oxyhemoglobin at pH 7.1 alpha chains are oxidized 9 times faster than beta chains (7).

Furthermore, ferricyanide is reported to oxidize alpha chains faster than beta chains in the fully reduced tetramer ($\alpha_2^{II}\beta_2^{II}$) (7).

Acknowledgement

The financial assistance of the U.S. Energy Research & Development Administration under Contract No. EY-76-C-02-3221 is gratefully acknowledged. We are very grateful to Prof. J. Peisach for helpful discussions.

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Table 1

Rate Constants for the Oxidation
of Individual Chains in Mixed State Hemoglobin Tetramers

	k_2	k_3	k_2/k_3
pH	$M^{-1} s^{-1}$	$M^{-1} s^{-1}$	
6.3	8.5×10^5	1.4×10^5	6.1
7.0	2.2×10^5	3.5×10^4	6.3
9.2	5.5×10^4	0.5×10^4	11

k_2 and k_3 are the different rate constants that can be obtained for the ferricyanide oxidation of two different one site reduced methemoglobins prepared by pulse radiolysis.

Legend to Figure

Fig. 1: The rate constants k_{obs}^2 and k_{obs}^3 for the reaction of one site reduced methemoglobins at pH 7, as a function of ferricyanide concentration.

○ $k_{\text{obs}}^2(\text{s}^{-1})$ ● $k_{\text{obs}}^3(\text{s}^{-1})$

Δ in the presence of $10^{-3}\text{M Fe}(\text{CN})_6^{-4}$