Nonequivalence of Chains in Hemoglobin Oxidation†

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ABSTRACT: The study of autoxidation of hemoglobin A followed by rapid chain separation revealed that the oxidation rates of α and β chains differ by a factor of ten. This difference was most evident at pH 6.5 but disappeared at pH 9.0. It is present but less pronounced in the case of isolated chains. Analogous results were obtained when Hb A was oxidized by $K_3[Fe(CN)_6]$. Lowering the oxygen pressure increased the rate of autoxidation, more so for β than for α chains. Two factors thus govern the oxidation rate: (1) intrinsic oxidizability of α being faster than β; (2) the presence or absence of ligand. Decreasing O_2 pressure affects the oxidation rate of β chains more than that of α chains; β chains thus appear to have a lower affinity for oxygen.

Only in its divalent state is heme able to bind oxygen reversibly. The half-life of heme in its divalent form in solution is very short, since it is rapidly oxidized to its trivalent form. The insertion of heme in the hydrophobic environment of the specific binding site of heme proteins decreases markedly its oxidation rate. Furthermore, red cells have enzymatic systems to reduce oxidized heme groups of hemoglobin (Hb). At all times a small proportion of the hemoglobin, ~1% (Paul and Demp, 1944; Wintrobe, 1967), is present as methemoglobin in the erythrocyte. This implies that also inside the red cell there is a constant oxidation of heme iron which must subsequently be reduced for the preservation of oxygen transport. Since the environment of the heme appears to be so important for the stability of the divalent form, it was suspected that the different hemoglobin chains would differ in their rate of oxidation. That this is the case for both isolated chains and tetrameric hemoglobins is shown in the present paper. Furthermore, the work presented here shows that the rates of autoxidation depend on both pH and oxygen pressure, analogous to the observations made by Brooks (1931, 1935) on whole ox blood.

Materials and Methods

General. All operations were carried out at 4° unless otherwise stated. All buffers contained 10^{-4} M EDTA. The chemicals used were of analytical grade.

Hemoglobin. Only freshly isolated hemoglobin was used. Hemoglobin A was isolated from freshly prepared dialyzed human hemolysate (15 g of Hb) (Winterhalter and Huehns, 1964) by chromatography on a column (25 × 1200 mm) of DEAE-Sephadex equilibrated with 0.05 M Tris-HCl buffer (pH 8.6). The column was eluted with a 2-l. linear gradient going from the equilibrating buffer to 0.05 M Tris (pH 6.5). The purity was checked electrophoretically and only fractions that were more than 99% pure were used for experiments.

Chains were isolated as described by Bucci and Fronticelli (1965). Complete removal of *p*-chloromercuribenzoate was checked as described elsewhere (Winterhalter and Colosimo, 1971). In the case of partially oxidized hemoglobin the sample was treated with CO for reaction with divalent heme and KCN in amounts sufficient to react with all methemoglobin present. In this case, rapid chain separation (3 hr) was achieved by a molar ratio of Hb tetramer to *p*-chloromercuribenzoate of 1:15.

Electrophoresis. For checking purity starch gel electrophoresis was used (Poulik, 1957). For the separation of chains obtained from partially oxidized hemoglobins (20 mg/ml) aliquots (25–100 μl) were applied to 7.5% polyacrylamide gels in a pH 8.3 Tris-glycine buffer which was saturated with CO immediately prior to the runs.

Oxidation Procedures. Autoxidation was carried out in 0.1 M phosphate buffer (pH 7.2) at 37°. Hemoglobin solutions or isolated chains (0.5 mM in heme) were sterilized by filtration through Millipore filters of 0.22-μ pore width. The samples were incubated in closed or open, bent, sterile test tubes. Aliquots were removed at intervals for methemoglobin determination and chain separation.

Chemical oxidation was carried out in 0.1 M phosphate buffer (pH 7.5) at 8°. In several experiments potassium ferricyanide was added to oxyhemoglobin in a ratio to give about 50% methemoglobin. The sample was then treated with CO and KCN and subsequently passed through a small column of Sephadex G-25 equilibrated with 0.1 M phosphate buffer (pH 6.0) containing 0.2 M NaCl. The rapid chain separation procedure was then carried out as described above.

Spectrophotometric Measurements. Spectra were obtained on a Beckman Acta V double beam spectrophotometer or alternately on a Beckman DB double beam spectrophotometer. Methemoglobin content was determined by the method described by Dubowski (1964). In the case of isolated chains which are unstable in the met form, a sufficient amount of KCN was added before oxidation to convert all the methemoglobin formed to the stable cyanmet form. The sample was allowed to react with CO just prior to recording the spectrum. Calibration curves for unknown mixtures of carbon monoxy and cyanmet chains were obtained by mixing known amounts of identical chains in their carbon monoxy and cyanmet forms for both types of chains. Oxygen equilibria were done as described by Rossi-Fanelli and Antonini (1958). Concentrations of hemoglobin on polyacrylamide gels were determined at 522 nm (an isobestic point of carbon monoxy and cyanmethemoglobin) with a Beckmann gel scanner.

By the method of least squares we fitted the observed autoxidation curves of hemoglobin A to a biphasic reaction consisting of a rapid initial reaction, followed by a slower second

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† From the Friedrich Miescher-Institut, CH-4002 Basel, Switzerland. *Received June 18, 1973.* This work is dedicated to Professor F. Leuthardt, Biochemisches Institut der Universität Zürich, on the occasion of his 70th birthday.

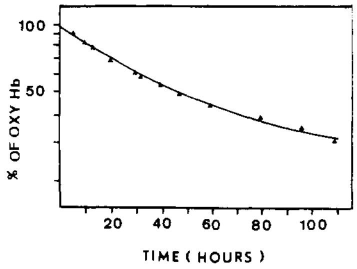

FIGURE 1: Autoxidation of Hb A at pH 7.2 and 37°. For details see text.

reaction with per cent autoxidation = $100 - 100(pe^{-\varphi t} + (1 - p)e^{-\sigma t})$ where φ and σ are constants of the two reactions and p is the percentage of the sites which are autoxidized rapidly. Since this fit had to be done by a nonlinear least-squares scheme, the calculation of confidence limits of the fitted parameters is very complex. We therefore calculated what changes of the parameters would double the variance for the measured values of the autoxidation curve and added these numbers in brackets.

Results

Autoxidation. Hemoglobin A showed a biphasic autoxidation curve (Figure 1). The initial fast rate constant was found to be $\varphi = 0.032$ ($\pm$0.002) hr^{-1}. The second phase of the reaction had a rate constant of $\sigma = 0.0037$ ($\pm$0.0006) hr^{-1}. The value for the percentage of fast reacting sites was $p = 52$ ($\pm$10) %.

For the autoxidation of isolated chains the rate constant for α chains was $\bar{\varphi} = 0.025$ ($\pm$0.003) hr^{-1} and that for β chains was $\bar{\sigma} = 0.009$ ($\pm$0.001) hr^{-1} at pH 7.2. However, because of the instability of isolated chains the experiments were performed at 25° (Figure 2).

In order to demonstrate which type of chain was the more rapidly autoxidizable, hemoglobin A was incubated until it was approximately 30% autoxidized and then the chains were separated as described under Materials and Methods. Figure 3 shows the calibration curve for mixtures of carbon monoxy and cyanmet chains and Figure 4 represents the spectra obtained from the α and β chains, respectively. It was calculated that the α chains had a cyanmet content of 53%, whereas only 5% of the β chains were in the cyanmet form. The remaining unsplit hemoglobin had 32% methemoglobin. It needs to be pointed out that in these experiments the yields of separated chains were always high and comparable for both chains. No significant amount of precipitate was observed.

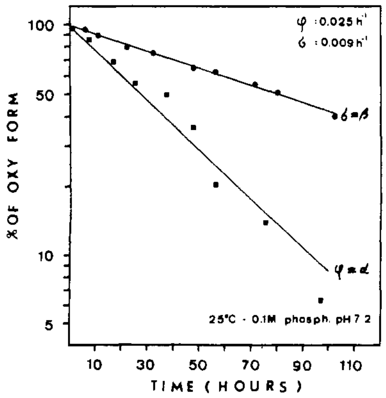

FIGURE 2: Autoxidation of isolated α (■) and β (●) chains at pH 7.2 and 25°.

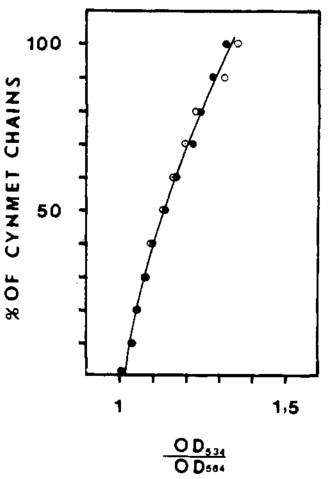

FIGURE 3: Calibration curve of α and β chains. Each point on this figure represents the ratio of optical density (OD) at 534 over 564 for a known mixture of cyanmet and carbon monoxy α (●) or β (○) chains.

Furthermore, it could be demonstrated that lowering the oxygen pressure to 57 and 40.5 mm, which induced no appreciable change in the oxy spectrum of the hemoglobin in the tonometer, increased the rate of autoxidation and the size of the rapidly oxidizing compartment (Figure 5). Chain separation revealed that the β chains were much more affected than the α chains (Table I).

Chemical Oxidation. Aliquots of hemoglobin were partially oxidized to about 50% and the chains separated. Also in these experiments it became apparent that the α chains oxidize preferentially.

Dependence of Oxidation on pH and Ionic Strength. The autoxidation at 37° and chemical oxidation with potassium ferricyanide at 8° were carried out at pH 6.5, 7.2, 8, and 9. As the pH increases, the rate of oxidation decreases. The autoxidation experiments were also carried out with and without 0.2 M NaCl at pH 7.2. High ionic strength resulted in a more rapid rate of oxidation (Figures 6 and 7).

Functional Properties of Partially Oxidized Hemoglobin. Figure 8 gives the values of n from the Hill equation as a function of oxidation. At approximately 55% oxidation, the value of n levels off at about 1.38. Also given in Figure 8 are the respective values of the logarithm $p(O_2)$ needed for half-

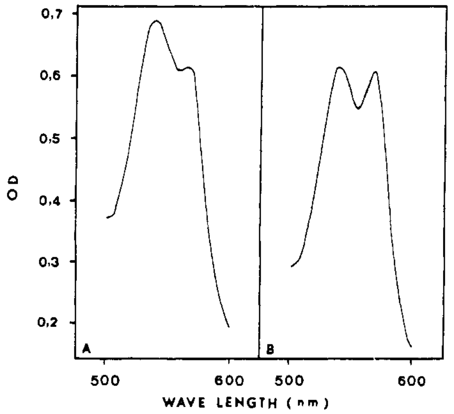

FIGURE 4: Spectra of α (A) and β (B) chain bands on polyacrylamide gel after autoxidation.

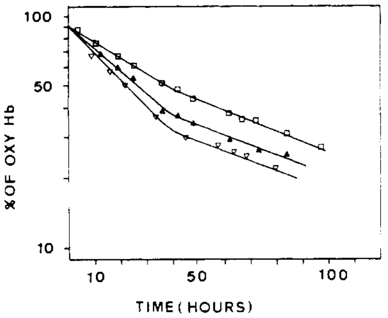

FIGURE 5: Autoxidation of Hb A (pH 7.2) under three different $p(O_2)$ values: (□) $p(O_2)$ = 160 mm; (▲) $p(O_2)$ = 57 mm; (▽) $p(O_2)$ = 40.5 mm; 37°.

saturation as a measure of the affinity for oxygen. In order to test for the influence of ligand on the oxidized chains on the ligand properties of the divalent chains CN^- ions were added. The *n* values and $p(O_2)^{1/2}$ thus obtained are also illustrated in Figure 8.

Discussion

As can be clearly seen from the data reported here, there is a large difference in the rate of autoxidation of α and β chains. The rate constants φ = 0.032 (±0.002) hr^{-1} for α and σ = 0.0037 (±0.0006) hr^{-1} for β differ almost by a factor of ten. This difference is present even in isolated chains when autoxidized at 25°, although to a lesser extent $\bar{\varphi}$ = 0.025 (±0.003) hr^{-1} and $\bar{\sigma}$ = 0.009 (±0.001) hr^{-1}.

For two reasons it is, however, difficult to compare results obtained on isolated chains directly to the observations in tetrameric hemoglobin. First, they were done by necessity at a different temperature and secondly the heme protein contact and probably the entire conformation in isolated chains differ from those of chains incorporated into tetramers (Winterhalter *et al.*, 1971).

In view of the fact that the yield of chains after separation of partially oxidized tetramers was almost quantitative and comparable for both chains, it seems unlikely that the spectral differences observed after chain separation can be accounted for by simply assuming preferential precipitation of one type of met chains over the other type. The study of the autoxidation reaction of Hb A_2 and F in our laboratory—not reported here—revealed that Hb A_2 has a similar autoxidation reaction to that of Hb A with a fast and a slow component, whereas Hb F autoxidizes in one phase with the rate of the reaction similar to that of the fast reacting component of Hb A and A_2. According to Ingram (1963) the γ chain, also rapidly autoxidizable, originated from the α chain through gene duplication, the β chain from the γ chain, and the δ chain in turn from the β chain. A decreased degree of autoxidation constitutes an advantage for the reducing economy of the cell. It is therefore tempting to speculate that β and δ chains would be an evolutionary improvement over α and γ chains.

TABLE I: Autoxidation of Hemoglobin A under Different $p(O_2)$.[a]

	% Oxidation			
$p(O_2)$ (mm)	α	Hb A	β	α/β
150	53	32	5	10.6
40.5	65	44	28	2.32

[a] Incubation (30 hr) at 37°.

In the α chains 18 amino acids and in the β chains 20 amino acids make contact with heme. Only 14 are identical amino acid residues on both chains.

It is clear that the different affinity of the two types of chains for heme cannot be responsible for the faster rate of oxidation, since the α chains have a higher affinity for heme (Winterhalter and Deranleau, 1967; Bunn and Jandl, 1968) than the β chains. The preferential oxidation of α chains will of course lead to oxidation intermediates similar to the ones described elsewhere (Brunori *et al.*, 1970) having the formula $\alpha_2{}^+\beta_2$. If the trivalent α chain is liganded to a CN^- ion, this compound has an *n* value of 1.2–1.3 (Brunori *et al.*, 1970; Haber and Koshland, 1969), a value similar to n = 1.2 found here after the addition of CN^- (Figure 8). The addition of CN^- firmly freezes the met chains in the *r* state, whereas without CN^- the met chains probably have a somewhat greater conformational flexibility. The observed influence of the added CN^- on the *n* values is thus in keeping with the general concept reached from kinetic data (Maeda and Ohnishi, 1971). The concept of preferential autoxidation of α chains and the appearance of oxidation intermediates also supply a rationale to the common observation that only 20% methemoglobin formation drastically alters the functional properties of hemoglobin solutions in both equilibrium and kinetic experiments since ~40% of the tetrameric molecules would be affected. Furthermore, the strong dependence of the rate of autoxidation on both pH and ionic strength of the solvent is of interest for choosing appropriate storage and chromatographic procedures for hemoglobin solutions.

It is important to point out that the rate of autoxidation of the slow component was *not* noticeably influenced by a change of pH from 6.5 to 9.0. The rate of the rapidly oxidiz-

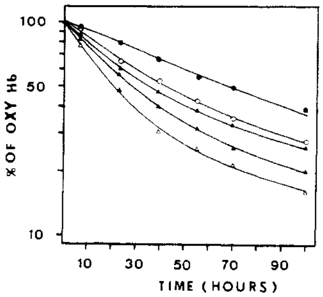

FIGURE 6: Autoxidation of Hb A at 37° and pH 9 (●), pH 8 (○), pH 7.2 (▲) without NaCl (upper curve), with 0.2 M NaCl (lower curve), and pH 6.5 (△).

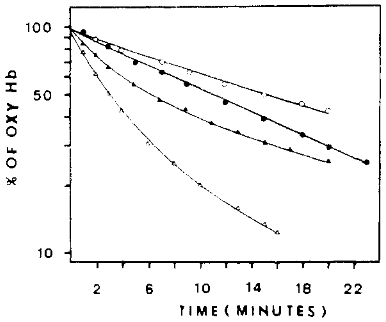

FIGURE 7: Oxidation of Hb A by potassium ferricyanide at 8° and pH 9 (○), pH 8 (●), pH 7.2 (▲), and pH 6.5 (△). The concentration of the oxidant was 1 M per each mole/liter of heme.

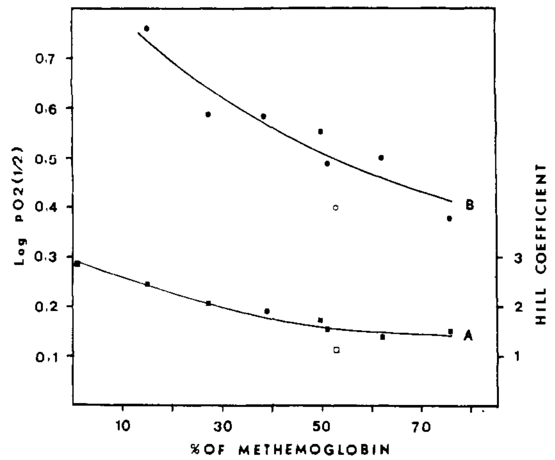

FIGURE 8: The relationship of cooperativity (■) and affinity (●), and the ratio methemoglobin/hemoglobin in a given solution: (□) and (○) represent, respectively, the cooperativity and affinity of a sample containing 53% cyanmethemoglobin.

able component to the contrary became slower with the increase of pH and was the same as the one of the slow component at pH 9.0. Also, chain separation after partial autoxidation at pH 9.0 revealed identical spectra for both types of chains.

The oxidation of hemoglobin by an oxidant, in our case potassium ferricyanide, gave similar results as autoxidation with preferential oxidation of α chains. The oxidation rates, however, very highly depend on the presence or absence of ligands on the hemes (E. Di Iorio, A. Mansouri, and K. H. Winterhalter, manuscript in preparation). Oxidation is roughly two orders of magnitude faster in deoxyhemoglobin than in oxyhemoglobin. In fact, lowering the oxygen pressure in a tonometer also resulted in an increased rate of autoxidation.

In conclusion it therefore appears that two mechanisms greatly influence the oxidation rate of the two types of chains: (1) the intrinsic rate of oxidation which is greater for α chains than for β chains; (2) the presence or absence of ligand. The fact that lowering the oxygen pressure to an extent which did not result in any appreciable change of the visible spectrum speeded up the autoxidation of β chains much more than the one of α chains seems to indicate that in oxyhemoglobin the β chains have a lower affinity for oxygen than the α chains.

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