A ROLE FOR CHLORIDE IN THE AUTOXIDATION OF HEMOGLOBIN UNDER CONDITIONS SIMILAR TO THOSE IN ERYTHROCYTES

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1. Introduction

It has been known for many years that hemoglobin in blood normally undergoes autoxidation at a rate of about 3% per day [1] but is returned to the active form by reductase and other systems. In some abnormal hemoglobins this autoxidation may be more extensive and/or more permanent leading to various forms of methemoglobinemia [2]. Although there have been some proposals concerning the mechanism of methemoglobin formation in specific instances [3] there is still very little understanding of the processes by which hemoglobin undergoes autoxidation [4]. However, our recent observation that a variety of anions are able to promote the autoxidation of hemoglobin through a proton assisted nucleophilic displacement of superoxide from oxyhemoglobin [5] may provide some insight into these physiologically important processes.

A number of reports have appeared indicating that both electrolyte in general [4] and chloride ion [6] in particular influence the rate of methemoglobin formation in blood. In addition there now seems little doubt that superoxide is present in erythrocytes in which normal autoxidation is occurring [7,8]. Chloride is well known [9] as the principal anionic constituent of both blood plasma (~ 0.12 M) and erythrocytes (~ 0.08 M) and our preliminary measurements [5] seemed to indicate that despite its low nucleophilicity chloride can act to slowly displace superoxide from HbO_2. It seems then not unreasonable to suppose that chloride ion plays an important role in the normal autoxidation of hemoglobin under physiological conditions. If the displacement mechanism is followed the presence of superoxide dismutase in erythrocytes is a reasonable consequence of the need to protect against the deleterious effects of the superoxide so produced. It may also be possible that the aging of stored blood in blood banks is in significant part due to lipid peroxidation or other reactions by the traces of superoxide that escape scavenging by superoxide dismutase.

We report here a more extensive examination of the role of chloride in causing autoxidation of HbO_2 under a range of conditions near to physiological with respect to temperature, pH, and kind and concentration of electrolytes present (Cl^-, $H_2PO_4^-$, HCO_3^-).

2. Experimental

Oxyhemoglobin was prepared by the method of Geraci et al. [10] and stored in solution at 4°C. This solution was stable under the conditions of storage for several days. The buffers were prepared from a solution containing 25.0 meq $NaHCO_3$ and 1.6 meq NaH_2PO_4 per liter which approximates the concentrations of the anions in erythrocytes [11]. The desired pH values were obtained by adjustment with either dilute perchloric acid or dilute sodium hydroxide using a Beckman Expandomatic Model SS-2 pH meter. All solutions were prepared from glass distilled water.

Reaction solutions were prepared by adding a weighed quantity of sodium chloride (or other

sodium salt) to a known volume of stock buffer solution which was adjusted to the required pH. These solutions were thermostated at the desired temperature (to ± 0.1°C) and when temperature equilibrium was established the reaction was initiated by addition of HbO_2 solution to a convenient concentration (in the range 20–50 μM). The reaction was followed by sampling the solutions at measured time intervals and determining the visible absorption spectrum (from 700–500 nm) on a Cary 17 spectrophotometer.

3. Results and conclusions

The reactions show (fig. 1) pseudo first order kinetics with dependence upon the concentration of oxyhemoglobin. It is, however, evident from the data in table 1 that the reaction rate is additionally dependent upon pH, temperature and electrolyte concentration. The linear hydrogen ion dependence (Expt. 1–3) is analogous to that observed previously with more powerful nucleophiles [5]. The Arrhenius plot, shown in fig. 2, of the temperature coefficient data (Expt. 4–6) gives an activation energy of 32 kcal/mole for the reaction. This very large activation energy accounts for the very slow autoxidation rate and for the remarkable stability of hemoglobin solutions stored at low temperatures. By contrast the more rapid reaction with azide is found to have an activation energy of 26.8 kcal/mole [12].

It is in the dependence of the reaction upon the kind and concentration of electrolyte present that chloride behaves differently from the more powerful nucleophiles (such as azide) reported upon previously [5]. Simple linear dependence upon the concentration of nucleophile is not observed with chloride. Although there is no doubt (table 1) that higher chloride concentrations mean more rapid metHb formation a general electrolyte effect as well as a specific chloride ion effect is apparent. The general

Table 1
Rate constants for the formation of metHb from HbO_2 under various conditions of temperature pH and electrolyte in bicarbonate–phosphate buffer

Expt. No.	pH	Temperature °C	Electrolyte Type	Electrolyte Concentration	μ^a	$k(M^{-1}\ hr^{-1})$
1	7.15	37.0	Cl^-	0.11	0.14	0.0052
2	7.40	37.0	Cl^-	0.10	0.13	0.0032
3	7.68	37.0	Cl^-	0.11	0.14	0.0018
4	7.40	34.0	Cl^-	0.10	0.13	0.0019
5	7.40	37.0	Cl^-	0.10	0.13	0.0032
6	7.40	40.0	Cl^-	0.10	0.13	0.0063
7	7.40	37.0			0.03	0.0002
8	7.40	37.0			0.13	0.0015
9	7.40	37.0			0.50	0.0192
10	7.40	37.0	Cl^-	0.06	0.09	0.0028
11	7.40	37.0	Cl^-	0.08	0.11	0.0030
12	7.40	37.0	Cl^-	0.13	0.16	0.0036
13	7.40	37.0	Cl^-	0.10	0.50	0.0229
14	7.40	37.0	Cl^-	0.20	0.50	0.0237
15	7.40	37.0	Cl^-	0.50	0.50	0.0324
16	7.40	37.0	F^-	0.10	0.50	0.0348
17	7.40	37.0	N_3^-	0.10	0.50	0.175

[a]Total ionic strength includes contribution from background buffer (0.03), sodium chloride and sodium sulfate required to make up the recorded ionic strength.

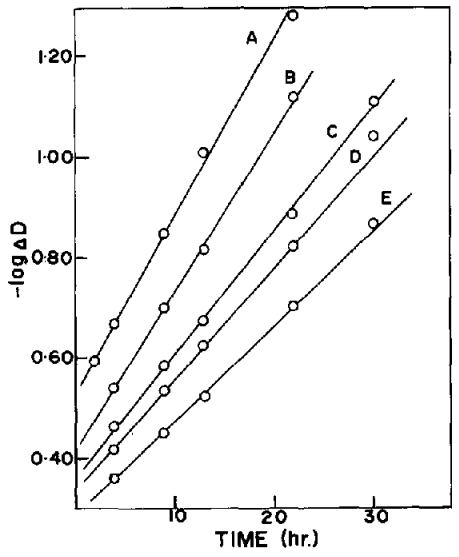

Fig. 1. Pseudo first order rate plots for the autoxidation of HbO_2 at $\mu = 0.5$. The electrolyte environment is made up to contain the electrolytes shown below together with sufficient sodium sulfate to bring the total ionic strength to 0.5. A) 0.1 M F^-; B) 0.5 M Cl^-; C) 0.2 M Cl^-; D) 0.1 M Cl^-; E) sodium sulfate only. The temperature was 37°C.

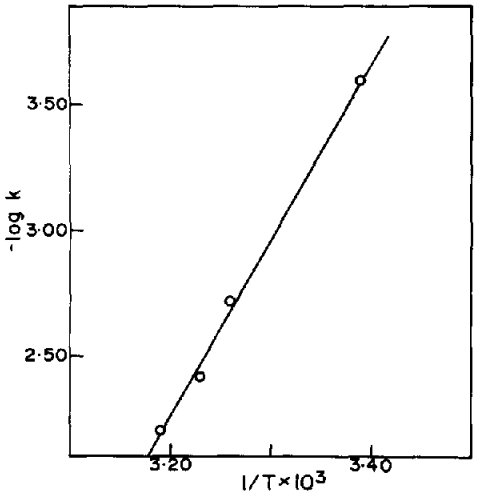

Fig. 2. Dependence of log K upon 1/T °K for the autoxidation of HbO_2 in the presence of 0.1 M Cl^- at a total ionic strength of 0.13.

electrolyte effect is observable when sulfate is the only anion present in the reaction mixture. The effect of this electrolyte alone falls off very rapidly as its total concentration decreases. This may be due to multiple, non specific binding of ions to the protein which gives rise to loss of tertiary structure, denaturation and eventual precipitation of the protein. At similar concentrations chloride ion is seen to be more effective than sulfate in producing autoxidation (compare 8 with 12 and 9 with 15). This may be due to chloride playing the dual role of general electrolyte and nucleophile which forces superoxide off the iron and becomes bound in its place. Support for this analysis comes from a comparison of the results from Expt. 2,9,13. The difference between the rate constants at $\mu = 0.5$ with and without 0.1 M chloride present (0.0037 hr^{-1}) is in good agreement with the value (0.0032 hr^{-1}) (see also ref. [6]) obtained in the presence of 0.1 M chloride as the sole electrolyte. If the general electrolyte effect of both sulfate and chloride is the same a general correction can be applied to the chloride data. When this is done a nearly linear dependence upon chloride emerges and the assignment of a specific nucleophilic role for chloride is strengthened. Thus, the weak nucleophile chloride is able to promote the autoxidation of HbO_2 by both the general electrolyte and direct displacement pathways, and at 0.1 M chloride the two pathways appear to make roughly equal contributions to the overall reaction. It is important to note that the result is the same regardless of which pathway is followed. Oxyhemoglobin is converted to methemoglobin with the concurrent production of superoxide (observed by reaction with ferricytochrome *c*). It was, unfortunately, not possible to unequivocally distinguish between acid metHb and metHbCl by the spectrophotometric technique employed. The spectra of the two complexes are very similar [13] and precipitation always occurred with these very slow reactions before 100% completion. There were however small intensity differences in the 630 nm band depending upon the concentration of chloride in the reaction mixture which suggested that chloride was bound under conditions where the displacement reaction predominated. The final two entries in table 1 show that as more powerful nucleophiles are employed for the promotion of the autoxidation reaction the general electrolyte effect becomes of diminished importance and the displacement reaction becomes predominant.

It seems quite clear that even with very weak nucleophiles the reaction involves the loss of superoxide from perturbed oxyhemoglobin. The perturbing forces are protonation and interaction with the electrolyte. The site of protonation is unknown but it seems reasonable, on the basis of the presence of the general electrolyte effect, that the bound oxygen is involved. The general electrolyte effect, could then have its origin in an electrolyte induced relaxation of the heme pocket allowing freer access of water to the oxygen binding site. The oxygen which had been perturbed by proton binding might then be susceptible to solvolytic removal as HO_2 with H_2O binding to iron in its place. The specific chloride effect would, in terms of the displacement mechanism, be the result of the energetically more favorable direct nucleophilic attack of the anion upon the perturbed (protonated)FeO_2 binding site.

The chloride mediated autoxidation of HbO_2 under conditions that approximate to physiological proceeds at about the right rate (5–7% in 24 hr) to account for the normally observed metHb formation in erythrocytes. This must mean that unexpectedly large amounts of superoxide are being continuously produced in erythrocytes. Indeed, superoxide pro-

duction need not be limited to erythrocytes for other oxygen carrying hemes (such as myoglobin) also undergo the electrolyte promoted autoxidation [12] demonstrated here for the chloride–hemoglobin couple. The superoxide would react readily with other molecules that it might encounter in the immediate environment, and the need to provide an efficient scavenging system for this very reactive by-product of autoxidation becomes readily apparent. However, even in the presence of superoxide dismutase superoxide may escape scavenging and react with lipids and other molecules. These reactions which could include epoxidation, peroxidation and polymerization may induce fragility and aging in cells, carcinogens in other tissues [14] and be generally important in tissue degradation processes.

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