THE MECHANISMS OF HEMOGLOBIN AUTOXIDATION

EVIDENCE FOR PROTON-ASSISTED NUCLEOPHILIC DISPLACEMENT OF SUPEROXIDE BY ANIONS

W. J. Wallace, J. C. Maxwell, and W. S. Caughey
Department of Biochemistry
Colorado State University
Fort Collins, Colorado 80521

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Summary. Human oxyhemoglobin (HbO_2) in the presence of excess nucleophile (e.g., N_3^-, SCN^-, F^-, Cl^-) is shown by visible and Soret spectra to form cleanly the oxidized metHb with the nucleophile as ligand. The rates, sensitive to pH and to both the concentration and the nucleophilicity of anionic nucleophile (N^-), follow the rate law: rate = $k[HbO_2][N^-][H^+]$. This autoxidation process thus appears to involve the nucleophilic displacement of superoxide from a protonated intermediate and can reasonably account for normal metHb formation in the erythrocyte where chloride can serve as the nucleophile. MetHb formation due to electron transfer agents (e.g. nitrite) which are normally not present can follow a different course such as direct electron transfer to bound dioxygen to form iron (III) peroxide. Abnormal amino acids or denaturation can provide increased access of nucleophile or electron transfer reactant and thus promote autoxidation.

Introduction

In the normal human erythrocyte Hb A undergoes autoxidation to a small extent with the metHb formed converted back to functional Hb by the methemoglobin reductase and other systems which keep metHb levels at about 1% of the total Hb present (1). Mechanistic details of metHb formation which are of clinical, as well as chemical, interest have been quite unclear.

There are many ways in which Hb could be oxidized to metHb but an autoxidation process with HbO_2 as an intermediate has been thought likely. For the conversion of HbO_2 to metHb either the loss of O_2^- (superoxide ion) to leave an iron (III) complex or addition of an electron to bound O_2 to give an iron (III) peroxide could be involved.

Recently, on the basis of infrared and other evidence, we concluded that there was strong covalent bonding between iron and bent, end-on dioxygen (i.e., $Fe{=}O{-}O$) and that such bonding precluded ready loss of O_2^- from HbO_2 (2,3). A description of the FeO_2 bonding as a fully developed iron (III)

superoxide ion couple (4,5,6) was shown to be unattractive in many respects (2). These arguments made the dissociative loss of O_2^- most unlikely. However, two recent reports presented evidence for superoxide generation during hemoglobin autoxidation (7). Also, there have been a number of reports that high salt concentrations enhanced the rate of HbO_2 autoxidation (8). Since the loss of O_2^- via a simple dissociative process is so unexpected, we examined the possibility that O_2^- could be displaced from HbO_2 by anionic nucleophiles (2). If oxygen is bound covalently, the displacement with anions should either not occur, or should occur only slowly, in consequence of the activation energy necessary to cause sufficient electronic rearrangement to allow dioxygen to separate from the complex as superoxide.

Methods and Materials. HbO_2 was obtained from human blood by the method of Geraci et al.(9). Solutions stored at 4°C were stable to denaturation and oxidation over a period of several days. Other chemicals were of reagent grade. Acetate buffer was used in the pH range from 4.5 to 5.8, maleate from 5.0 to 6.7, phosphate from 6.2 to 8.3 and borate from 8.0 to 9.3. The buffer concentrations were generally held high (i.e.,~0.5M) to reduce the effect on the pH of the addition of the basic anionic nucleophiles. Some experiments were run at lower buffer concentrations (down to 0.01M) without noticeable change in the reaction.

Reaction solutions were prepared by dissolving a weighed amount of salt in a known volume of the appropriate buffer, the pH of the solution measured, the Hb solution added, and the resulting solution transferred to a 1.0 cm cuvette. The reaction was followed by observing either repetitive scans over the visible spectrum at measured intervals of time or the change in absorbance with time at 577 nm using a Cary 17 spectrophotometer in both instances.

Results. HbO_2 was found to react with aqueous solutions of a number of salts in high concentration to form metHb complexes. The series of spectra shown in Figure 1 represent the spectral changes that occur when sodium azide reacts with HbO_2 at pH 5.55 in 0.5 M acetate buffer. The isosbestic points

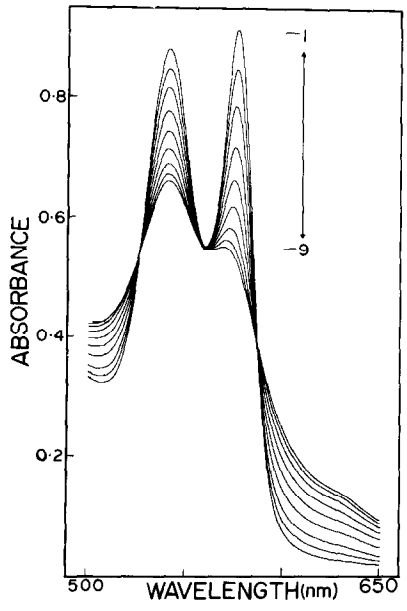

Figure 1. Spectral changes occurring with time when .076M sodium azide reacts with .59 x 10^{-4}M oxyhemoglobin at pH 5.55 in 0.5M acetate buffer at 24°C. 1 - 0.0 min, 2 - 2.0 min, 3 - 4.0 min, 4 - 7.0 min, 5 - 11.0 min, 6 - 15.0 min, 7 - 20.0 min, 8 - 30.0 min, 9 - 45.0 min.

at 586 nm and 526 nm indicate that only a single product is formed and the spectrum (of the final product) indicates that it is the metHbN_3 complex (10). Similar observations were made for the reactions with the other salts. In each instance identification of the reaction product as the metHb-anion complex was made spectrophotometrically. These reactions did not occur in the absence of anion binding to the iron (III) in the metHb product. In contrast to findings by others in other systems (11), the rate of autoxidation of HbO_2 was not observed to be influenced by the degree of oxygenation nor were rate differences between α and β chains noted.

For this family of reactions plots of log $(D-D_\infty)_{577}$ against time were linear (Figure 2) and the pseudo first order rate constants obtained from these plots are given in Table 1 as k_{app}. These rate constants are seen to be linearly dependent upon both anion and hydrogen ion concentration and the rate law is represented as equation 1.

$$R = k\,[FeO_2][N^-][H^+] \qquad \text{----------1}$$

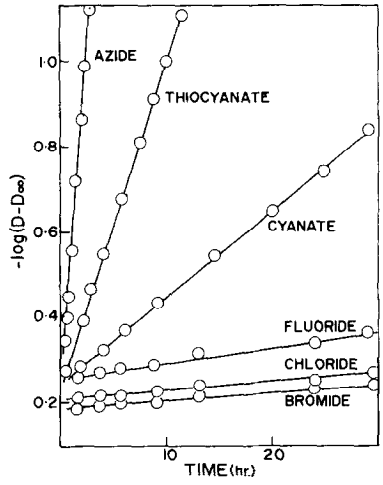

Figure 2. Plot of $-\log(D-D_\infty)$ at 577 nm as a function of time for the reaction between HbO_2 and the anions azide (.075M), thiocyanate (.069M), cyanate (.073M), fluoride (0.20M), chloride (0.20M), bromide (0.20M)

When HbCO was treated with azide at a concentration of 0.1 M, spectral changes occurred so slowly that if the displacement reaction actually were occurring leading to the loss of CO^-(?), it was slower by a factor of 10^6 to 10^8 than the similar displacement on HbO_2.

Conclusions. It is clearly possible to carry out a reaction on HbO_2 in which the oxygen is replaced by an anionic ligand and the iron is converted to the ferric state. Since none of the replacing groups was a potential electron acceptor, the electronic charge must have been carried off with the oxygen as superoxide. A reaction in which superoxide is displaced by an anionic ligand provides a simple pathway for the autoxidation of Hb, and also a reason for the presence of superoxide dismutase, in erythrocytes.

Through the pH range in which the reactions were studied the concentrations of undissociated acids were so small ($<10^{-3}$M) compared with the free anions (~0.1 to 0.5 M) that attack almost certainly occurred via the anion. Furthermore, if the free anion is the attacking group, the order of reactivity follows the nucleophilicity of the entering group $N_3^->SCN^->OCN^->F^->Cl^-$. Indeed the pseudo second order rate constants (k_{app}/N^-) shown in the last column of Table 1

Table I

Kinetic Parameters for the Displacement of Dioxygen (as Superoxide) from Oxyhemoglobin† by Anionic Nucleophiles at 24°

Nucleophile		Buffer		Rate Constants		
Type	Concn(M)	Type	Concn(M)	pH	$k_{app} \times 10^2$ (min^{-1})	$k^1 \times 10^2$ ($min^{-1}\ M^{-1}$)*
Azide	.162	Maleate	0.1	5.7	9.07	56.2
	.093		0.1		5.27	56.7
	.042		0.1		2.35	56.1
Azide	.076	Phosphate	0.5	6.4	0.85	11.4
	.565		0.01	7.4	0.50	0.95
	.504	Borate	0.1	8.3	0.071	0.14
	.484		0.1	9.3	0.010	0.022
Azide	.075	Acetate	0.5	5.0	12.2	239
Thiocyanate	.076				2.58	34.0
Cyanate	.062				0.67	10.8
Fluoride	.114				0.13	1.14
Fluoride	.200	Maleate	0.1	6.4	0.0063	0.032
Chloride	.200				0.0040	0.020
Bromide	.200				0.0030	0.015

*$k^1 = k_{app}/[N^-]$.

†Initial oxyhemoglobin concentrations were always within the range (0.4 - 1.0) x 10^{-4}M. The rate constant for a first order reaction is independent of the initial concentration of the reactive species.

(as k') fit in a quite acceptable way the two parameter correlation for nucleophilic displacement reactions suggested by Edwards (12). These observations suggest that the reaction proceeds by way of a proton assisted nucleophilic displacement of superoxide from HbO_2 by anionic entering groups.

It is useful to compare the displacement of superoxide from HbO_2 with the displacement of common anions such as azide, cyanate and fluoride from their

metHbX complexes. In contrast with the superoxide displacement which is slow and requires very high concentrations of displacing reagent, these latter reactions are very rapid and require only stoichiometric amounts of the displacing reagent (8,13,14). There is then a greater dependence on bond formation in the reactions of oxyhemoglobin and this reflects an energy requirement in the superoxide displacement reaction that is not present in the displacement of other ligands. We attribute this to the electronic rearrangement necessary when the iron (II) dioxygen complex transforms, under the influence of the incoming group, to iron (III) complex and the superoxide ion. It is this activation energy for the displacement reaction that is mainly responsible for the resistance that HbO_2 shows to autoxidation via a superoxide loss mechanism. Either a high concentration of nucleophile or a very strong nucleophile would then be required to cause the reaction to proceed rapidly. Under physiological conditions the potential displacing ligands are OH^-, Cl^-, HCO_3^-. If the displacing ligand is hydroxide, the autoxidation remains slow under all conditions since increasing the ligand (OH^-) concentration will result in a compensating decrease in $[H^+]$. Chloride and bicarbonate are both poor nucleophiles but chloride is sufficiently reactive that with a normal human erythrocyte concentration of ca. 0.1 M (15) it could account for much, if not all, of the normal autoxidation rate.

Electron donors small enough to penetrate to the bound oxygen, such as nitrite, are not normally present under physiological conditions. Hence the stability of HbO_2 under physiological conditions can be related both to the pH and to the absence of suitable autoxidation agents (either nucleophiles or electron carriers). Those oxyhemoglobins that do undergo ready autoxidation must have a heme pocket that is sufficiently modified to allow reactants ordinarily present or a group on the protein (e.g. a tyrosine) to act by one of the mechanisms described. It will be of interest to examine in much greater detail the autoxidation reactions of abnormal as well as normal hemoglobins.

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