[Chem. Pharm. Bull. 29(5)1382—1389(1981)]

Factors Influencing the Autoxidation of Hemoglobin A

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(Received November 1, 1980)

Autoxidation of hemoglobin A incubated at 20° or 37° over a period of 24 hr was affected by several factors. Oxidation of oxyhemoglobin (HbO_2) and deoxyhemoglobin (DeoxyHb) was faster as the pH value decreased. Allosteric effectors (IHP and EDTA) and reducing agents (ascorbic acid and cysteine) promoted the proton-assisted oxidation of HbO_2 to produce methemoglobin (MetHb) and/or precipitates. Metal ions (Fe^{2+}, Fe^{3+}, Cu^{2+} and Hg^{2+}) had a stimulatory effect at pH 7 and 5.5 on the production of MetHb or precipitates from both HbO_2 and DeoxyHb. The Cu^{2+}- and Hg^{2+}-catalyzed oxidations of HbO_2 were dramatically suppressed by EDTA, while those catalyzed by Fe^{2+} and Fe^{3+} were accelerated by EDTA. The Fe^{2+} (and Fe^{3+})-EDTA complexes were potent inducers of the oxidation in the presence of oxygen, while EDTA blocked the Fe^{2+}-catalyzed oxidation of DeoxyHb. The oxidations catalyzed by all these metal ions were suppressed by ascorbic acid and cysteine. The Fe^{2+} (and Fe^{3+})-ascorbate (and cysteine) complexes effectively deoxygenated HbO_2.

Keywords——hemoglobin A; proton-assisted autoxidation; IHP; EDTA; ADP; ascorbic acid; cysteine; Fe^{2+}, Fe^{3+}, Cu^{2+}, Hg^{2+}, Fe^{2+} (and Fe^{3+})-EDTA; Fe^{2+} (and Fe^{3+})-ascorbate; Fe^{2+} (and Fe^{3+})-cysteine

Reversible oxygenation of hemoglobin of physiological importance occurs only with hemoglobin [Fe(II)] and not with methemoglobin [Fe(III)].[1] The autoxidation of hemoglobin refers to its transformation into methemoglobin in the presence of oxygen.[2,3] The location of the heme [Fe(II)] in a hydrohobic crevice coordinated to the globin protects the ferrous ion and thereby enhances the stability of hemoglobin.[4,5] In the erythrocyte, a low level of methemoglobin is maintained by its enzymatic reduction into hemoglobin under normal conditions.[1]

Nevertheless, the autoxidation of hemoglobin occurs at a significant rate under anomalous conditions.[6] In some human abnormal hemoglobins this autoxidation may be more extensive and/or more permanent, leading to methemoglobinemia.[2,3] The autoxidation may be involved in α and β thalassemias and has been recognized to be the first event in the formation of Heinz bodies.[7] There have been several reports on the autoxidation of isolated mammalian and non-mammalian hemoglobins, in which the effects of protons,[6] oxygen,[6,8] the superoxide anion and hydrogen peroxide,[9–14] several nucleophiles,[15,16] and transition metal ions[13,18,19] were described.

In the present work we investigated the autoxidation of human normal hemoglobin (hemoglobin A), and elucidated the effects of varying the concentration of protons, transition metal ions with or without chelating agents, or reductants.

Experimental

Materials——Human oxyhemoglobin (HbO_2) was purified by passage through a Sephadex G-25 (fine) column in order to remove inorganic contaminants according to the methods previously reported.[20] Concentration of heme of HbO_2 was determined by absorbance measurements based on an extinction coefficient at 576 nm of 16500.[21] HbO_2 solution was treated with an approximate five fold excess of potassium ferricyanide,[19] and the spectrum was recorded as that of standard methemoglobin (MetHb) in buffers (pH 4.5, 5.0 and 5.5) to calculate the molecular extinction coefficients at these pH values. The spectrum of MetHb thus prepared could not be recorded in buffers at pH 3.5 and 4.0 owing to spontaneous formation of a pre-

cipitate. Inositol hexaphosphate (IHP) (phytic acid from corn, type IV, sodium salt) was obtained from Sigma Chemical Company. Disodium ethylenediamine tetraacetate dihydrate (EDTA) (Wako Pure Chemical Industries, Ltd.), disodium adenosine 5′-diphosphate (ADP) (Yamasa Shoyu Co., Ltd.), L-ascorbic acid (Kanto Chemical Co., Inc.) and L-cysteine (Ajinomoto Co., Inc.) were products of the indicated companies. Zinc chloride, cadmium chloride, lead chloride, ferrous chloride tetrahydrate, ferric chloride hexahydrate, cupric sulfate pentahydrate and mercuric chloride were of reagent grade. The buffers used were 0.1 M sodium acetate (pH 3.5, 4.0, 4.5, 5.0 and 5.5), 0.1 M sodium potassium phosphate (pH 7.0) and 0.05 M Tris-HCl (pH 7.0, 8.0, 9.0 and 10.0).

Treatment of Hemoglobin——HbO_2, deoxyhemoglobin (DeoxyHb) and MetHb (30—50 μM) were treated under the indicated conditions. DeoxyHb was prepared by deoxygenation of HbO_2 with nitrogen gas, and treated under anaerobic conditions by the use of a cuvette attached to a Thunberg tube. Visible spectra were recorded with a Shimadzu UV-200S double beam spectrophotometer equipped with a thermostatically controlled apparatus.

Results

Oxyhemoglobin(HbO_2) prepared from normal human red cells was treated in buffers (pH 3.5—10.0) at 20° and at 37°. No spectral changes were observed in tris buffers (pH 8—10) over a period of 24 hours, but the spectra in acetate and phosphate buffers (pH 3.5—7.0) changed in a pH-dependent manner. The spectra in the acidic ragnes showed a time-dependent decrease in absorbances at 576 (α band) and 540 nm (β band) nm and an increase in absorbance at 630 nm due to methemoglobin (MetHb) formation with excellent isosbestic points at 523 and 587 nm. Representative spectral changes at pH 5.5 and 37° are illustrated in Fig. 1A. Increases in absorbance at 630 nm were faster as the pH value decreased, and faster at 37° than at 20° (Fig. 2). The conversion of 40 μM HbO_2 at 37° into MetHb was less than 10 (pH 7), 21 (pH 5.5), 35 (pH 5) or 50% (pH 4.5) after 60 min, and reached 48% (pH 7) or 74% (pH 5.5) after 24 hours. The conversion at 20° was less than 10 (pH 7) or 24% (pH 5.5) after 24 hours. The first-order kinetics of the autoxidation at 37° and at pH 4.5, 5.0 and 5.5 are presented in Fig. 3. Deoxyhemoglobin (DeoxyHb), prepared by deoxygenation of HbO_2, was treated at 37° under anaerobic conditions. The spectrum was little changed at pH 7 and pH 5.5 during 60 min, whereas the spectrum at pH 3.5 was transformed into that of MetHb within 10 min.

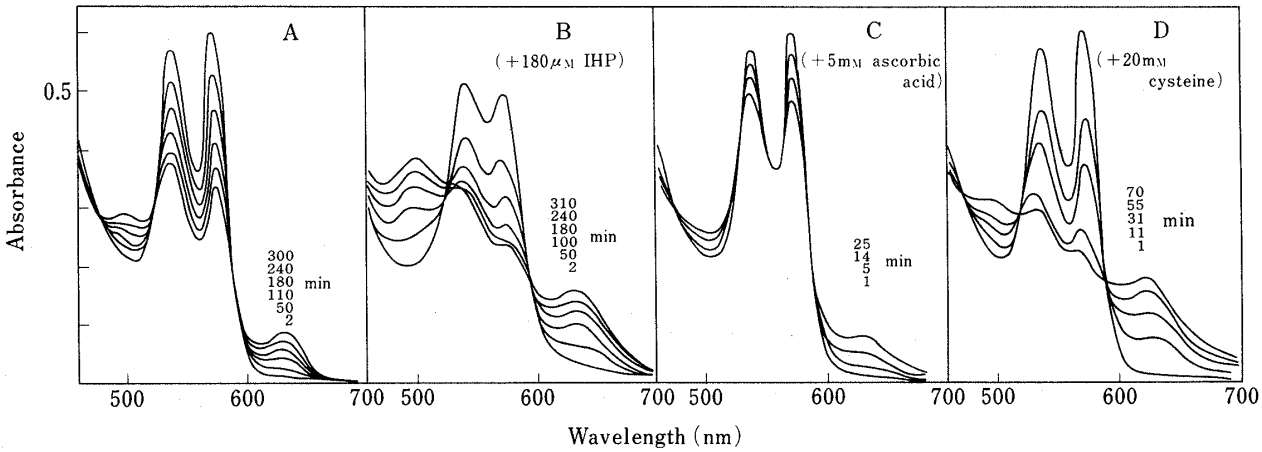

Fig. 1. Spectral Changes of Hbo_2 (38 μM) in 0.1 M Acetate Buffer (pH 5.5) at 37°

Prolonged treatments in the presence of IHP, ascorbic acid or cysteine, for periods longer than those indicated in the figure produced marked precipitates.

HbO_2 was treated at pH 5.5 and 37° in the presence of inositol hexaphosphate (IHP), an allosteric effector of hemoglobin[22] (Fig. 1B). Addition of IHP caused spontaneous large spectral changes accompanied by decreases in absorbance at the α and β bands and an increase in absorbance at around 560 nm. The rate of increase in absorbance at 630 nm with time

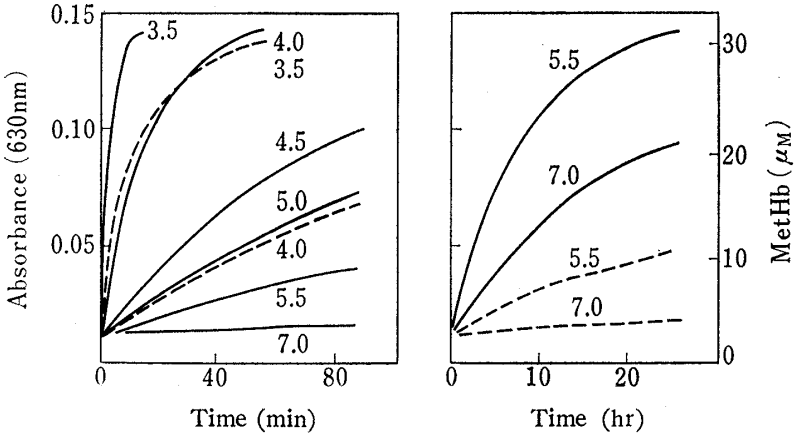

Fig. 2. Time Courses of Increase in Absorbance at 630 nm of 40 μM Hbo_2 treated in 0.1 M Acetate Buffer (pH 3.5—5.5) and in 0.1 M Phosphate Buffer (pH 7) at 37° (——) and 20° (----)

The numerals in the figure indicate the pH value.

was much higher than in the absence of IHP, but the isosbestic points were not clear during the reaction, probably because of the gradual formation of a precipitate. When HbO_2 was treated at pH 7 with IHP, spontaneous spectral changes due to the conformational change from R to T structure[23] were less, and the rate of the increase in absorbance at 630 nm with time was significant but much less than at pH 5.5. In the strongly acidic region, such as pH 3.5 and 4.0, addition of IHP induced precipitation.

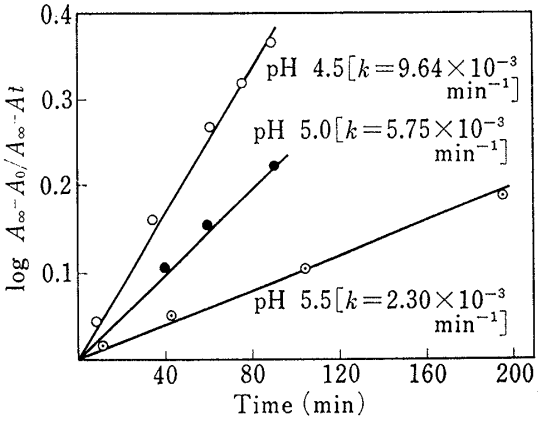

Fig. 3. First-order Rate Plots for the Autoxidation of 40 μM Hbo_2 in the Buffer at 37°

When HbO_2 was treated with 5 mM EDTA or 4.4 mM ADP at pH 7 and 5.5 and at 37° over a period of 24 hours, MetHb formation was little influenced. In contrast, when HbO_2 was treated with EDTA or ADP at pH 3.5 and at 37°, the rate of increase in absorbance at 630 nm was markedly enhanced.

HbO_2 was treated with 5 mM ascorbic acid at pH 7, 5.5 and 3.5. The mixture incubated at pH 7 and 20° showed gradual spectral changes similar to those of the control over a period of 3 hours, but showed larger changes than those of the control after 10 hours, indicating enhanced formation of MetHb and a precipitate. The mixture incubated at pH 7 and 37° produced a greenish precipitate within 3 hours. The spectral changes of HbO_2 treated with the acid at pH 5.5 and 20° showed no significant spectral changes compared with the control over a period of 24 hours, but the mixture treated at pH 5.5 and 37° gradually produced MetHb and a precipitate (Fig. 1C). When HbO_2 was treated with the acid at pH 3.5 and 37°, the increase in absorbance at 630 nm was also enhanced. The effect of the acid on the proton-assisted autoxidation seemed greater as the pH value increased.

In the treatment of DeoxyHb with ascorbic acid at pH 7 and 5.5 at 37° under anaerobic conditions, no spectral changes were observed during 60 min. Treatment of MetHb with the acid at pH 7 and at 37° under anaerobic conditions gave DeoxyHb within 60 min, whereas treatment of MetHb with the acid under aerobic conditions gave HbO_2 within 60 min and produced a precipitate upon prolonged treatment.

HbO_2 was treated with 20 mM cysteine at pH 7, 5.5 and 3.5. Transformation of HbO_2 by the agent seemed greater at pH 7 than at pH 5.5 and 3.5. The mixtures incubated at pH 7 and 5.5 at 20° for 10 hours showed gradual spectral changes similar to those of the controls. The mixture incubated at pH 7 and 37° for 3 hours produced a precipitate, accompanied by spectral changes showing maxima at 619 and 679 nm. Brief incubation of the mixture at pH 5.5 and 37° produced MetHb and a precipitate (Fig. 1D), and prolonged treatment (more than 3 hours) produced large amounts of precipitate with an absorption maximum at 619 nm. When HbO_2 was treated with cysteine at pH 3.5 and 37°, oxidation of HbO_2 was not affected. DeoxyHb was unaffected by treatment with cysteine at pH 7 and 5.5 at 37° under anaerobic conditions for 60 min. The treatment of MetHb with cysteine at pH 7 and 37° under anaerobic conditions gave DeoxyHb within 60 min, while treatment of MetHb with the agent under aerobic conditions gave a turbid solution exhibiting maxima at 539, 574, 619 and 679 nm after 4—20 hours.

The effects of metal ions such as Zn^{2+} ($ZnCl_2$), Pb^{2+} ($PbCl_2$), Cd^{2+} ($CdCl_2$), Fe^{2+} ($FeCl_2$), Fe^{3+} ($FeCl_3$), Cu^{2+} ($CuSO_4$) and Hg^{2+} ($HgCl_2$) on the autoxidation of HbO_2 were investigated. When HbO_2 was treated in 0.05 M tris buffer (pH 7) at 37° with each of the metal ions, Fe^{2+}, Fe^{3+}, Cu^{2+} and Hg^{2+} induced spectral changes with a precipitate, while Zn^{2+}, Pd^{2+} and Cd^{2+} did not influence the autoxidation even at 2 mM. On treatment of HbO_2 with 1.3 mM Fe^{2+}, 0.2 mM Fe^{3+} or 10 μM Cu^{2+} over a period of 2 hours, the absorbance at 630 nm gradually increased, accompanied by the formation of a precipitate. Hg^{2+} at 0.5 mM concentration produced a white precipitate. Precipitation could not be prevented by the use of lower concentrations of these ions. When Fe^{2+} and Fe^{3+} were combined with 5 mM EDTA, no precipitates were produced and the spectral changes indicated that MetHb was the only product. EDTA did not block MetHb formation by these ions. Formation of MetHb and a precipitate by Cu^{2+} was completely suppressed by EDTA. It was quite unexpected that the combination of Fe^{2+} and Fe^{3+} with ascorbic acid or cysteine gave neither MetHb nor precipitates but caused deoxygenation.

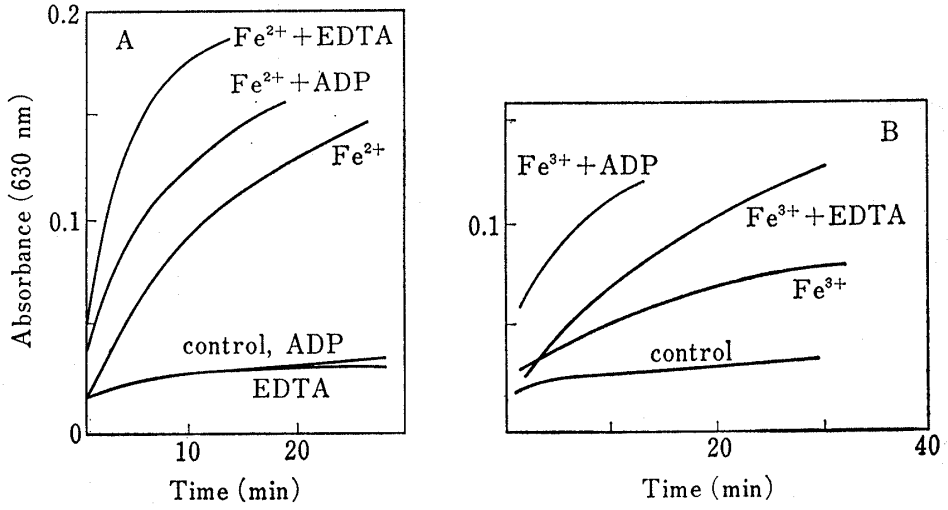

Fig. 4. Time Courses of Increases of Absorbance at 630 nm of 40 μM HbO_2 treated in 0.1 M Acetate Buffer (pH 5.5) at 37°

Concentrations were: 1.3 mM $FeCl_2$, 0.2 mM $FeCl_3$, 5 mM EDTA and 4.4 mM ADP. All the spectra showed characteristic isosbestic points for the conversion into MetHb.

HbO_2 was treated with Fe^{2+}, Fe^{3+}, Cu^{2+} and Hg^{2+} at pH 5.5 and 37° for 60 min. Treatment of HbO_2 with 1.3 mM Fe^{2+} markedly promoted the formation of MetHb with excellent isosbestic points (Fig. 4A). Addition of EDTA and ADP promoted the formation of MetHb by Fe^{2+}. Treatment of HbO_2 with Fe^{2+}-ascorbic acid (Fig. 5A) or Fe^{2+}-cysteine (Fig. 5B) caused spectral

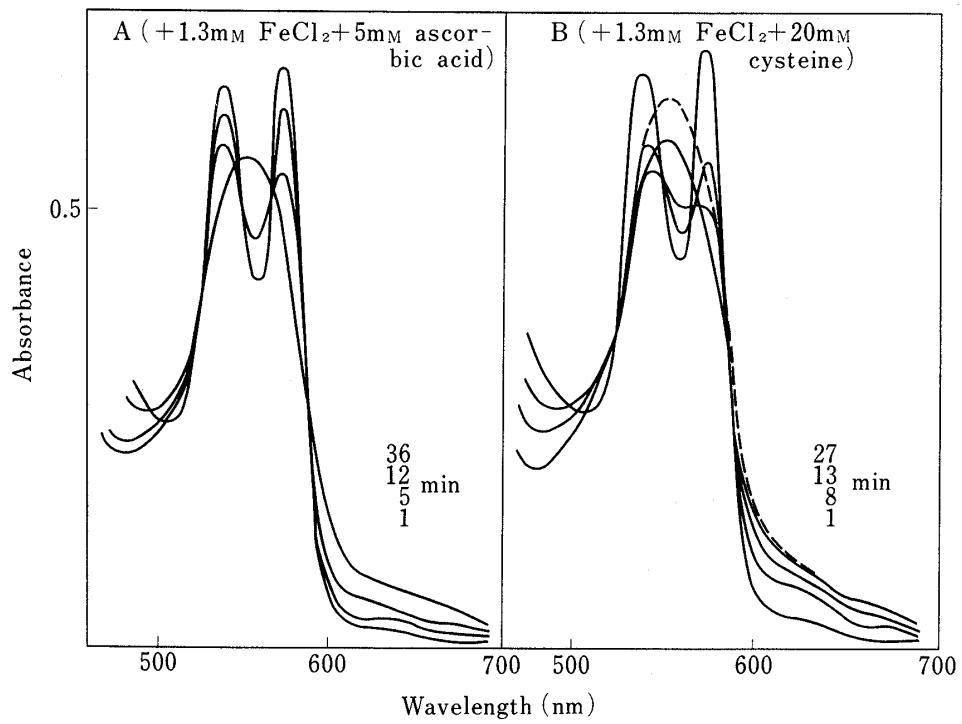

Fig. 5. Spectral Changes of HbO_2 (40 μM) in 0.1 M Acetate Buffer (pH 5.5) at 37°

The dotted line in B indicates that the treatment was performed in the presence of 2 mM KCN for 12 min.

changes indicating that deoxygenation took place. This was quite unexpected, since ascorbic acid (Fig. 1C), cysteine (Fig. 1D) and Fe^{2+} each induced MetHb when HbO_2 was separately treated with one of them under the same conditions. As regards Fe^{2+}-cysteine (Fig. 5B), cyanide showed the same spectral changes, indicating that MetHb was not an intermediate of DeoxyHb formation. Treatment of HbO_2 with 0.2 mM Fe^{3+} induced MetHb formation, and in this case EDTA and ADP also promoted MetHb formation (Fig. 4B). When HbO_2 was treated with Fe^{3+}-ascorbic acid or Fe^{3+}-cysteine, deoxygenation was apparent after incubation for more than 70 min. Treatment of HbO_2 with 10 μM Cu^{2+} produced MetHb readily, and the transformation was completely inhibited by EDTA. Combined treatment with ADP, ascorbic acid or cysteine accelerated the formation of MetHb or a precipitate. When HbO_2 was treated with Hg^{2+}, gradual formation of MetHb and a precipitate was observed.

When HbO_2 was treated with the metal ions at pH 5.5 and 20° for 60—100 min (Fig. 6), HbO_2 was gradually transformed into MetHb with excellent isosbestic points, without any accompanying precipitates. The concentrations of the metal ions were the highest that did not produce any precipitates under the conditions used. EDTA was stimulatory with Fe^{2+}, partially inhibitory with Fe^{3+}, and completely suppressive with Cu^{2+} and Hg^{2+}. ADP was stimulatory with Fe^{3+} and partially inhibitory with Fe^{2+} and Cu^{2+}. Ascorbic acid was completely suppressive with Fe^{2+} and Fe^{3+} and partially suppressive with Cu^{2+}. Cysteine completely blocked the reactions of Fe^{2+}, Fe^{3+}, Cu^{2+} and Hg^{2+}. All of these chelating and reducing agents were inert when HbO_2 was treated with each of them alone.

DeoxyHb was treated with Fe^{2+}, Fe^{3+} and Cu^{2+} at pH 5.5 and 37° under anaerobic conditions. Representative profiles of the spectral changes of DeoxyHb treated with Fe^{2+} are presented in Fig. 7A, showing its transformation into MetHb. Fe^{2+}, Fe^{3+} and Cu^{2+} were active in the formation of MetHb from DeoxyHb, causing increases of the absorbance at 630 nm, while EDTA, ascorbic acid, cysteine and Fe^{2+}-EDTA were inert (Fig. 7B). EDTA suppressed Fe^{2+}-catalyzed oxidation under anaerobic conditions.

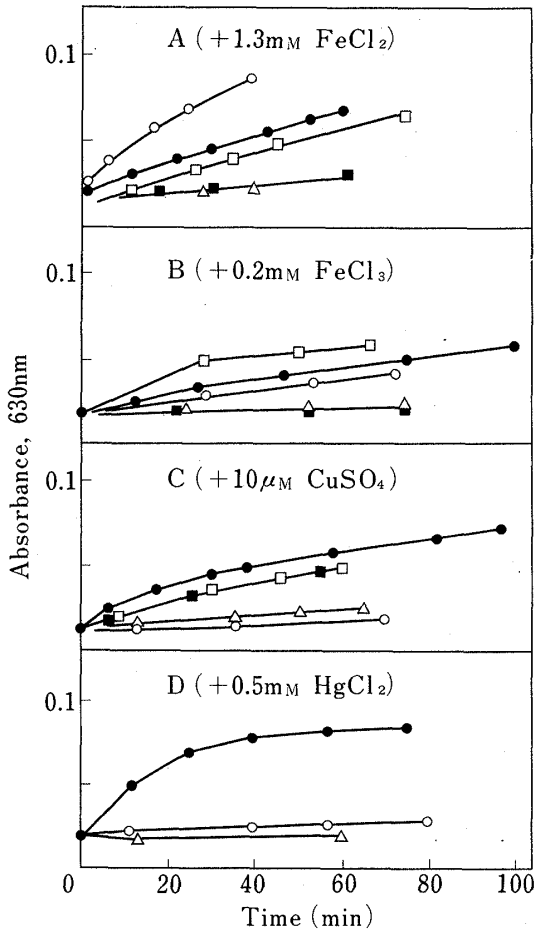

Fig. 6. Time Courses of Increase of Absorbance at 630 nm of 40 μM Hbo_2 treated in 0.1 M Acetate Buffer (pH 5.5) at 20°

○, +5 mM EDTA; □, +4.4 mM ADP; ■, +5 mM L-ascorbic acid; △, +20 mM cysteine. All the spectra showed characteristic isosbestic points for the conversion into MetHb. Spectra of HbO_2 solutions without metal ions were unchanged during the period.

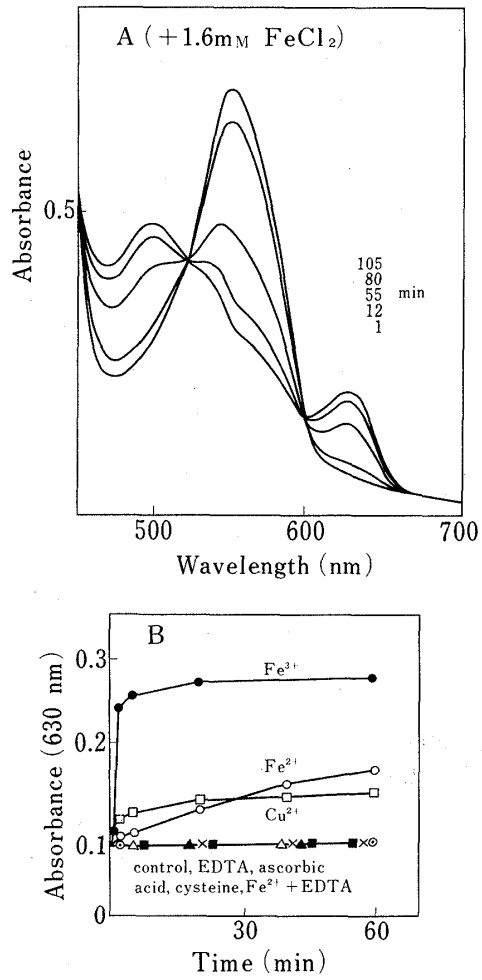

Fig. 7. Transformation of 50 μM DeoxyHb at pH 5.5 and 37° under Anaerobic Conditions

Concentrations were: 1.6 mM $FeCl_2$, 0.25 mM $FeCl_3$, 12.5 μM $CuSO_4$, 6.3 mM ascorbic acid and 25 mM cysteine.

Like Fe^{3+} and Cu^{2+}, Fe^{2+} produced neither DeoxyHb nor HbO_2 when it was incubated with MetHb at pH 5.5 and 37° for 60 min.

Discussion

It has been well documented that autoxidation of hemoglobin occurs under anomalous conditions,[6–19] but the details have not been elucidated. Thus, several factors that may affect the oxidation of hemoglobin A were systematically investigated.

The oxidation of HbO_2 under aerobic conditions was accelerated by an increase of proton concentration, as has been described by Brooks[6] The proton-assisted autoxidation of HbO_2 may be explained in terms of the release of superoxide from the superoxoferrihemoglobin complex,[24] as illustrated in the following scheme, because the superoxide is a weak acid having a pKa value of 4.8.[25] The released

$$\text{Fe[II]}\cdot O_2 \longrightarrow \text{Fe[III]}\cdot O_2^- \xrightarrow{H^+} \text{Fe[III]} + HO_2$$

superoxide may catalytically induce the formation of MetHb by interaction with HbO_2, as

shown previously.[13] Furthermore, DeoxyHb was also transformed into MetHb in the strongly acidic range under anaerobic conditions. The transformation might be explained by the intramolecular oxidation of Fe[II] to Fe[III] based on the conformational changes of the hemoglobin. Thus, the proton-assisted oxidation of hemoglobin might be initiated not only by release of the superoxide but also by conformational changes of the hemoglobin molecule.

IHP, an allosteric effector of HbO_2 which converts the R state into the T state,[22] enhanced the proton-assisted autoxidation, indicating that the conformational changes of HbO_2 played an important role in this process. EDTA and ADP also promoted the autoxidation at pH 3.5; similar conformational changes may be involved, since EDTA is an active allosteric effector.[26] This effect of EDTA is not in accord with earlier observations[13,18] that EDTA suppressed the autoxidation, and that contaminating metal ions activated the autoxidation.

Reducing agents such as ascorbic acid and cysteine accelerated the autoxidation of HbO_2 to produce MetHb and/or a greenish precipitate, the rate being greater at pH 7 than at pH 5.5. The electron-donating capability of these agents must have contributed to the abstraction of the superoxide anion from the superoxoferrihemoglobin complex of HbO_2, leading to the formation of MetHb and hydrogen peroxide. These agents also reduced MetHb to produce DeoxyHb or HbO_2, but were inactive on DeoxyHb. The results are consistent with earlier observations.[3,27–30]

Metal ions such as Fe^{2+}, Fe^{3+}, Cu^{2+} and Hg^{2+} promoted the proton-assisted autoxidation, while Zn^{2+}, Pd^{2+} and Cd^{2+} were inert. The ions promoted not only the autoxidation of HbO_2 but also the transformation of DeoxyHb, but did not affect the reduction of MetHb. It is conceivable that Fe^{3+} and Cu^{2+}, both being electron accepting agents, may cause the oxidation of Fe[II] in the presence and absence of oxygen,[13,18] but Fe^{2+}, an electron donating agent, may cause the transformation of HbO_2 into MetHb but not oxidize DeoxyHb. However, the results on Fe^{2+} are not in accord with this view. Thus, the results may be explained in terms of conformational changes of hemoglobin induced by Fe^{2+} rather than its electron donating character. The metal ion may combine with the globin molecule to induce conformational changes and then cause the transformation of Fe[II] to Fe[III] by intramolecular electron transfer.

The effects of chelating agents such as EDTA and ADP on the metal-catalyzed oxidation of HbO_2 were not uniform. EDTA suppressed the effects of Cu^{2+} and Hg^{2+} but rather stimulated Fe^{2+} and Fe^{3+} actions. Inhibition of Cu^{2+}-catalyzed oxidation by EDTA was previously demonstrated by Rifkind.[18] Fe^{2+}-EDTA and Fe^{3+}-EDTA complexes may be converted into the peroxide form in the presence of oxygen, and the peroxide might then accelerate the oxidation of HbO_2 at pH 5.5 and 37°. This assumption is supported by the results that the effects of Fe^{2+} on the oxidation of DeoxyHb were completely inhibited by EDTA and that the Fe^{2+}-EDTA complex was inert in the absence of oxygen. The enhancement of the effects of Fe^{2+} and Fe^{3+} by ADP at pH 5.5 and 37° might be explained similarly.

The effects of ascorbic acid and cysteine on metal-catalyzed oxidation were quite unexpected. Although the reducing agents were potent promotors of the autoxidation, they suppressed the metal-catalyzed reaction. At pH 5.5 and 20° they suppressed the effects of Fe^{2+}, Fe^{3+}, Cu^{2+} and Hg^{2+}, while at pH 7 or 5.5 and at 37° Fe^{2+}-ascorbate, Fe^{2+}-cysteine, Fe^{3+}-ascorbate and Fe^{3+}-cysteine deoxygenated HbO_2 to produce DeoxyHb. In the treatments with Fe^{2+}-cysteine, the use of the cyanide-trapping technique excluded the possibility that MetHb was produced as an intermediate and was then reduced to DeoxyHb. Thus, these combinations must have directly acted on HbO_2 to deoxygenate it without affecting the ferrous state of hemoglobin.

In conclusion, the autoxidation of hemoglobin A is very complex. It is influenced by oxygen, pH, allosteric effectors, reductants, metal ions, combinations of metal ions with chelating agents and combinations of metal ions with reductants. Many of these factors may be present in erythrocytes containing hemoglobin and in cell-free preparations of hemoglobin.

Therefore, investigations of the oxidation of hemoglobin should be carried out and interpreted with great care.

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