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ENHANCED OXIDATION OF BIS(3,5-DIBROMOSALICYL) FUMARATE α–α CROSS LINKED HEMOGLOBIN BY FREE RADICALS GENERATED BY XANTHINE/XANTHINE OXIDASE

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ABSTRACT

The xanthine/xanthine oxidase reaction produces reproducible amounts of oxygen-derived free radicals that oxidize human oxyhemoglobin (Hb). We monitored the kinetics of the oxidation of stripped Hb (sHb), purified HbA_0 and α–α cross-linked Hb (HbXL99α) at [Hb] in the 5 to 150 μM (heme) range. For increasing [Hb], the oxidation halftime ($t_{1/2}$) increased for all Hbs, but $t_{1/2}$ was always less for HbXL99α than for HbA_0 and sHb. Such feature was attributed to the lower affinity for O_2 of HbXL99α and may represent a serious problem for use of this Hb as blood substitute.

INTRODUCTION

The cytotoxicity of free plasma hemoglobin (Hb) involves free radicals [1,2]. Hb is known to generate free radicals during autooxidation [3,4] and to increase through the Fenton reaction the peroxidation of fatty acids initiated by free radicals generated by external sources [5-7]. Here, we focus on Hb as *target* of oxygen-derived free radicals (ODFR) generated by xanthine (XAN) and xanthine oxidase (XO). The main

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features of this approach are: 1) The amount of ODFR is modulated and reproducible because they are produced stoichiometrically by an enzymatic system; 2) The primary target of ODFR is distinguished from secondary targets such as peroxidation of lipids; 3) The time course of this reaction is faster than that of Hb autooxidation; 4) The effects of ODFR are easily monitored with spectrophotometric methods.

We characterized this model monitoring the spectral changes of HbO_2 when mixed with XAN/XO assuming the mechanism drawn in Fig.1 (unbalanced equations). We show that ODFR oxidize HbO_2 in a concentration-dependent fashion with an action likely directed towards the heme. The Hb derivative cross-linked between the α chains by bis(3,5-dibromosalicyl) fumarate (HbXL99α) appeared more susceptible than stripped Hb (sHb) and HbA_0 to the oxidation by ODFR perhaps for its lower O_2 affinity.

MATERIALS AND METHODS

Materials. The medium contained 50 mM K_2HPO_4, 1 mM ethylenediaminetetraacetate (EDTA), 0.5 mM XAN, 20 mM KCN, pH 7.3 at 37_C. Dimethylthiourea (DMTU, Aldrich), XO (20 U/mL, cow milk), superoxide dismutase (SOD, 5,000 U/mg, bovine erythrocytes), catalase (260,000 U/mL, beef liver), and cytochrome c (Boehringer Biochemia) were used without further treatment.

We prepared sHb from freshly drawn blood of non-smoker donors (HbCO<2%) [8]. Total [Hb] was determined by the met-cyanide method [9]. MetHb was obtained mixing HbO_2 with 20% molar excess solid $K_3Fe(CN)_6$ for 15 min at room temperature [8]. HbA_0 was purified by anion exchange high-performance liquid chromatography [10]. HbXL99α was obtained reacting HbA_0 with 3,5-bis-dibromosalicyl-fumarate [11].

Methods. Visible and UV spectra were obtained with a DU-70 (Beckman Instruments Inc., Fullerton, CA). Kinetics were recorded at 37 C by a DW-2a (American Instrumentation Co., Silver Spring, MD) dual wavelength spectrophotometer operating at 577-600 nm (3 nm slit opening). The 1-cm path length quartz cuvette was filled with 1 mL of air-saturated buffer ([O_2]_0.2 mM [12]) and appropriate amounts of HbO_2 were added to it. The reaction was started by adding 100 mU/mL XO to

OEC

$HbO_2 \rightleftharpoons Hb + O_2$

$Hb + H_2O_2 \xrightarrow{\cdot OH} metHb \xrightarrow{KCN} CNmetHb$

XO   SOD

$XAN \longrightarrow Urate + O_2^{\cdot -} \longrightarrow H_2O_2$
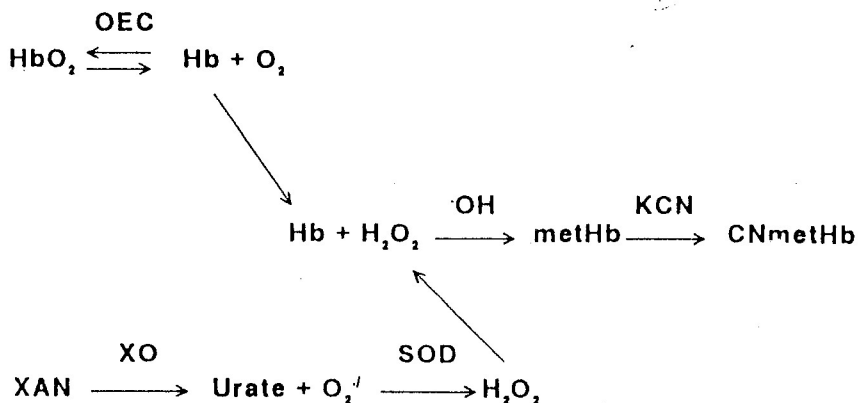
FIGURE 1. Scheme of the mechanisms assumed in this work (unbalanced reactions). Abbreviations: OEC, oxygen equilibrium curve; SOD, superoxide dismutase; XO, xanthine oxidase.

the mixture. The rate of $O_2^{\cdot -}$ production by XAN/XO was measured mixing 100 μM cytochrome c ($E_{550\ nm}$=24.3 mM^{-1} cm^{-1} in the reduced form) with 100 mU/mL XO and 0.5 mM XAN [13]. The Hb-O_2 affinity was measured at pH 7.4, 37°C (Hemox oxygen equilibrium curve analyzer).

RESULTS

Generation of ODFRs and Hb spectra. Under the selected conditions, $O_2^{\cdot -}$ was generated steadily at 0.045 mM/min for 2 min without initial lag phases. The oxidation of HbO$_2$ (all types) by 0.5 mM XAN or urate in the absence of XO was <0.25 μM/min (0.8%/min) and <0.6 μM/min (2%/min), respectively. The oxidation rate was not limited by shortage of XAN nor O$_2$, which was regenerated during the reaction (Fig.1). KCN was added to the medium to convert metHb to CNmetHb.

Spectra analysis in the visible and UV regions (not shown) indicated that HbO$_2$ was oxidized to CNmetHb. The absorbance change at 700 nm, index of aspecific protein denaturation [14], was <0.003 absorbance units/min under all conditions.

Kinetics. The oxidation of 50 μM/L sHb (Fig.2) was typically sigmoidal with maximal rate (V_{max})=22.7±2.1 μM/min (mean±SD, n=10).
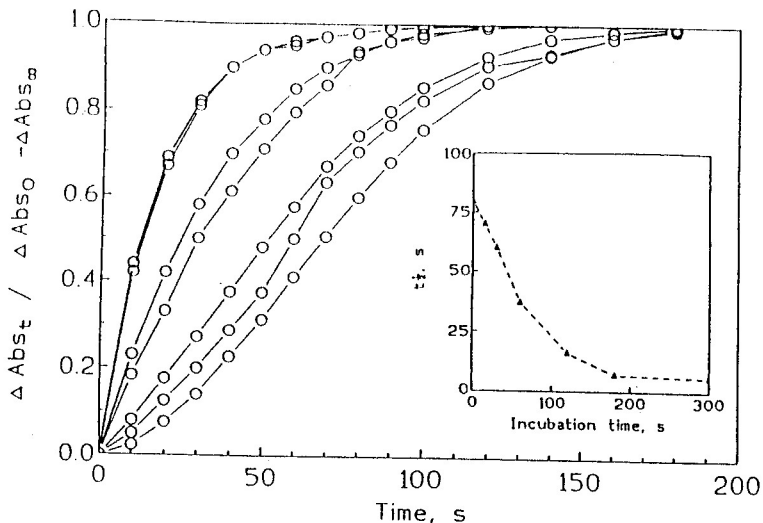
FIGURE 2. Kinetics of the oxidation of 50 μM stripped HbO_2 by pre-incubated mixtures of XAN/XO (from right to left, 0, 15, 30, 60, 120, 180 and 300 s). Each point represents the mean of 4 to 6 experiments (2% average S.D., not shown for clarity). $\Delta Abs = Abs_{577} - Abs_{600}$. Insert: Half-time ($t_{1/2}$) of the reaction vs the corresponding incubation time.

In the presence of CO, V_{max} was 0.7 ± 0.3 μM/min (n=5, p<0.0001). When sHb was added to pre-incubated XAN/XO (0 to 300 s) the sigmoidal shape disappeared and the oxidation halftime ($t_{1/2}$) decreased as function of the incubation time (Fig.2).

The oxidation of sHb was indistinguishable from that of HbA_0. Fig.3 shows the increase of $t_{1/2}$ at increasing [Hb] in the 5 to 150 μM range. At all concentrations, $t_{1/2}$ for HbXL99α was significantly less than for HbA_0.

In the presence of 50 mM DMTU, scavenger of $\cdot OH$, V_{max} reduced to 15.1 ± 1.2 μM/min (n=6, p<0.0001). In the presence of 2000 U/ml SOD, scavenger of $O_2^{\cdot -}$, V_{max} increased by 7-9% (p=NS). The effect of catalase, which quickly decomposes H_2O_2, was not tested for the inhibition of this enzyme by KCN, but experiments in the absence of KCN demonstrated that catalase prevents the oxidation of HbO_2 (not shown).
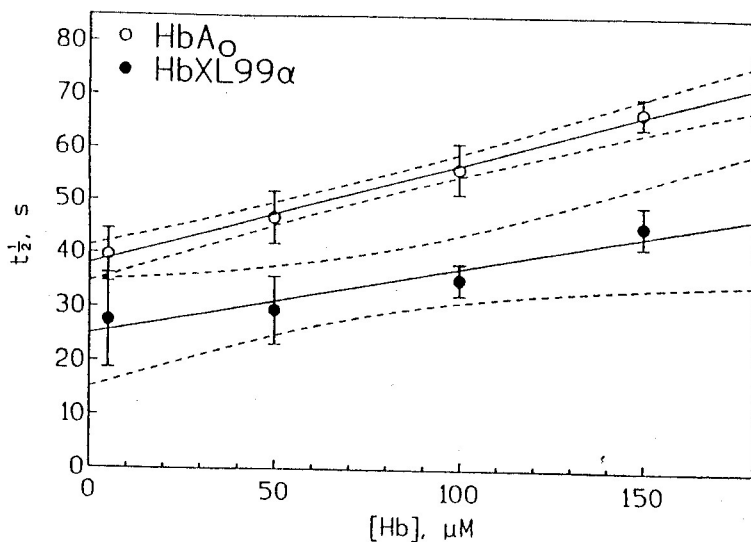
FIGURE 3. Halftime ($t_{1/2}$) of the oxidation of HbXL99α and HbA$_0$ (virtually the same as stripped Hb) as function of [Hb]. Dashed lines are the 95% confidence limits. The differences were significant at the p=0.03, 0.002, 0.0005 and 0.0005 levels (Student's t-test for unpaired data) at the four [Hb]'s.

The P$_{50}$'s of stripped Hb, HbA$_0$ and HbXL99α were 10.0, 11.5 and 30.0 mmHg, respectively, pH 7.4.

DISCUSSION

The XAN/XO system generates reproducible and accurate amounts of ODFRs that oxidize HbO$_2$ by specific mechanisms which are faster than HbO$_2$ autooxidation [6]. The oxidation rate depended on [Hb] for all Hb's, but HbXL99α was always oxidized faster than sHb and HbA$_0$.

Kinetic data fit a model where the first product of XAN/XO (O$_2$·$^-$) is unable to oxidize HbO$_2$, but slowly dismutes to H$_2$O$_2$ which is a strong oxidant. Several observations support this view: 1) When HbO$_2$ was added to pre-incubated mixtures of XAN/XO, the sigmoidal shape of the oxidation curve disappeared and the oxidation was faster; 2) SOD slightly

increased the oxidation rate presumably accelerating the formation of H_2O_2 [15-17]; 3) Catalase prevented the oxidation; 4) DMTU slowed significantly the oxidation rate scavenging the $\cdot OH$ radical.

The dependence of the oxidation rate on [Hb] could be related to the Hb tetramer splitting into dimers and to different reactivities of tetramers and dimers. For a dissociation constant of 0.001 mM [18], the tetrameric form accounts for 92.2% and 64.2% of total Hb at [Hb]=150 and 5 μM, respectively. However, the presence of $\alpha-\alpha$ covalent linkage in HbXL99α should have prevented splitting in this Hb, but nevertheless the kinetics of HbXL99α depended on [Hb] in the same way as HbA$_0$ and sHb, indicating that it is unlikely that the Hb tetramers and dimers have different reactivities in this model.

The heme appeared the elective site of action of ODFR because the oxidation was nearly abolished when O_2 was replaced by CO, consistently with the slow rate of CO dissociation from HbCO [19]. The faster oxidation of HbXL99α with respect to that of HbA$_0$ and sHb may thus be explained hypothesizing that the overall reaction is limited by the release of O_2 from HbO_2 reflecting different Hb-O_2 affinities of the various tested Hbs.

These data are in essential agreement with the faster autooxidation rate of HbXL99α with respect to HbXL82β and HbA [20]. However, we suggest that it is not only during storage that HbXL99α must be protected from oxidation, but also when infused into a patient, because this Hb is particularly susceptible to oxidation by circulating oxidative factors, especially those arising from reperfusion of ischemic tissues.

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