Production of Superoxide from Hemoglobin-Bound Oxygen Under Hypoxic Conditions

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ABSTRACT: By low temperature electron paramagnetic resonance we have detected the formation of a free radical signal during incubation of partially oxygenated hemoglobin at 235 K. The observed signal has $g_{\|} = 2.0565$ and $g_{\perp} = 2.0043$, consistent with the previously reported values for superoxide. The presence of additional EPR signals for oxygen-17 bound hemoglobin, with $^{(O17-O17)}A_{\perp} = 63$ G and $^{(O17-O16)}A_{\perp} = 94$ G under identical conditions, confirms the presence of a radical containing two nonequivalent oxygens as required for a superoxide in magnetically inequivalent environments. The superoxide radical has not previously been directly detected during hemoglobin autoxidation because of its rapid dismutation. Our ability to follow the formation of superoxide for more than 15 min is attributed to its production in the hydrophobic heme pocket where dismutation is slow. The enhanced production of this free radical at intermediate oxygen pressures is shown to coincide with enhanced rates of hemoglobin autoxidation for partially oxygenated intermediates. The formation of superoxide in the heme pocket under these conditions is attributed to enhanced heme pocket flexibility. Greater flexibility facilitates distal histidine interactions which destabilize the iron−oxygen bond resulting in the release of superoxide radical into the heme pocket.

The structural and molecular basis for cellular and tissue injury in oxygen deprived cells continues to be a problem relevant to medical practice (Halliwell & Gutteridge, 1990). Research is critically needed to define the site (of generation), kind, magnitude, and causative effects of free radicals emanating from hypoxic cells (Rifkind et al., 1992). In many situations the defense of the cells to oxygen deprivation takes the form of switching to reductive metabolic pathways requiring less energy. The hypoxic behavior of mammalian erythrocytes, which have a lesser metabolic potential due to loss of cellular organelles (nucleus, mitochondria, microsomes, etc), has not received much attention. Circulating erythrocytes are a constant source of superoxide owing to the well known tendency of hemoglobin to autoxidize (MacDonald, 1994). Under normoxic conditions, the intraerythrocytic cellular detoxicants (enzymes and antioxidants) are in dynamic equilibrium with cellular oxidants, so that reactive oxygen species (O_2^-, H_2O_2, etc), which are constantly generated intracellularly, are for the most part neutralized (Jaffe, 1964). However, evidence obtained shows that under hypoxic conditions leakage of superoxide does occur (Rifkind et al., 1989). This leakage has been explained by the unusual dependence of hemoglobin autoxidation on the partial pressure of oxygen. Instead of the rate increasing with the concentration of the oxidizing agent, namely, oxygen, a dramatic increase takes place when the oxygen pressure decreases (Rifkind et al., 1989). Therefore, even under normal conditions, as the blood passes through the capillaries and the pO_2 drops, the rate of superoxide production increases. This oxidative stress will of course be exacerbated whenever the organism is in a more hypoxic state which can result from circulatory problems and respiratory distress. An increased flux of superoxide radicals under reduced oxygen pressures thereby becomes a contributing factor to hypoxic tissue damage.

The superoxide radical is much less reactive than many other free radicals but is a precursor of other reactive oxygen species including hydrogen peroxide and hydroxyl radicals. In addition, the relatively long lifetime of the superoxide radical facilitates reaction at even remote sites sensitive to either oxidation or reduction by superoxide (Afanas'ev, 1991). Previous studies on myoglobin and later extended to hemoglobin have implied that autoxidation proceeds by an electron transfer from deoxygenated Fe(II)-heme via the porphyrin or the protein to an outer-sphere oxygen not coordinated with the heme (Abugo & Rifkind, 1994; Wallace et al., 1982; Dickerson et al., 1993; Brantley et al., 1993). In the present study we show that hypoxic autoxidation of hemoglobin involves predominantly an inner-sphere mechanism with an electron transferred from Fe(II) to bound oxygen leading to oxidized hemoglobin and the superoxide radical. This process may be facilitated by enhanced heme pocket flexibility under hypoxic conditions, which results in the distal histidine functioning as an endogenous nuclophile, with the iron-bound dioxygen functioning as an electron acceptor.

These studies have been possible because of our ability to directly detect the superoxide produced during autoxidation. This detection has generally not been possible previously (Goldberg et al., 1979; Knowles et al., 1969) because

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of the spontaneous dismutation or generation of secondary radicals.

By using low temperature electron paramagnetic resonance (EPR), we were able to detect the superoxide radical evolving from the hemoglobin-bound oxygen which is trapped in the hydrophobic heme pocket and therefore unable to dismutate.

MATERIALS AND METHODS

The red blood cells obtained from the healthy participants of the Baltimore Longitudinal Study on Aging were washed three times in phosphate buffered saline (PBS), pH 7.4, containing 0.1 mM EDTA, to remove buffy coat and plasma. The cells were lysed by freeze-thawing followed by centrifugation at 18 000 rpm for 30 min to remove the membranes. Hemoglobin A_o was obtained from the Letterman Army Institute of Research at the Presidio of San Francisco and stored at liquid nitrogen temperatures. Similar results were obtained with A_o and with fresh lysates.

For the preparation of partially oxygenated hemoglobin samples, two different concentrations (7.38 and 0.07mM) of hemoglobin were deoxygenated in a Labconco controlled glove box to specified partial pressures of oxygen and equilibrated by gentle rocking. At each pO_2, aliquots from the more concentrated sample were transferred to 4 mm OD quartz EPR tubes and quickly frozen in liquid nitrogen inside the glove box. Aliquots from the dilute sample were transferred to cells with ground glass joints for measurements in a visible spectrophotometer (Perkin Elmer Lambda 6) to determine the oxy, deoxy, and methemoglobin levels and to determine the rate of autoxidation (Abugo & Rifkind, 1994).

Hypoxic oxygen-17 enriched HbO_2 samples were prepared using specially designed spectroscopic cells and EPR tubes which contained a bulb near the top, to facilitate gas−liquid equilibration. These cells were connected through ground glass joints and rotoflo valves to a volume calibrated 2000 mL ballast, which was connected through a three-way ground glass stopcock to the vacuum system. To the third port in the three-way stopcock, 0.05 L of 85% enriched gaseous oxygen-17 (Isotec Inc., OH) cylinder was connected. After the samples were completely deoxygenated, the oxygen-17 cylinder was connected to the 2000 mL ballast. By the connection of other known volumes in parallel with the sample tubes, the partial pressure of oxygen was adjusted to about 5 mm Hg, which is required for 30−60% oxygenation of hemoglobin. After equilibration, the rotoflo valves were closed, and the samples were disconnected from the ballast and vacuum system. The EPR sample was then frozen and stored at 77 K until spectra were recorded. For comparison purposes, a natural abundance oxygen sample was prepared in the same way with the 0.05 L container filled with 100% natural abundance oxygen.

EPR spectra were measured at 8−10 K using an IBM ER-200D-SRC spectrometer with 100 kHz modulation and an Air Products Model LTD-3-110 liquid transfer Heli-Tran cryogenic unit with an APD-E temperature controller. Incubation of EPR samples was done in a Cryotrol Cryobath (CB-80, Neslab Instruments, Inc., NH) maintained at 235 K.

RESULTS

Figure 1 shows the effect of incubation at 235 K on the EPR spectra obtained at 8 K for a partially oxygenated

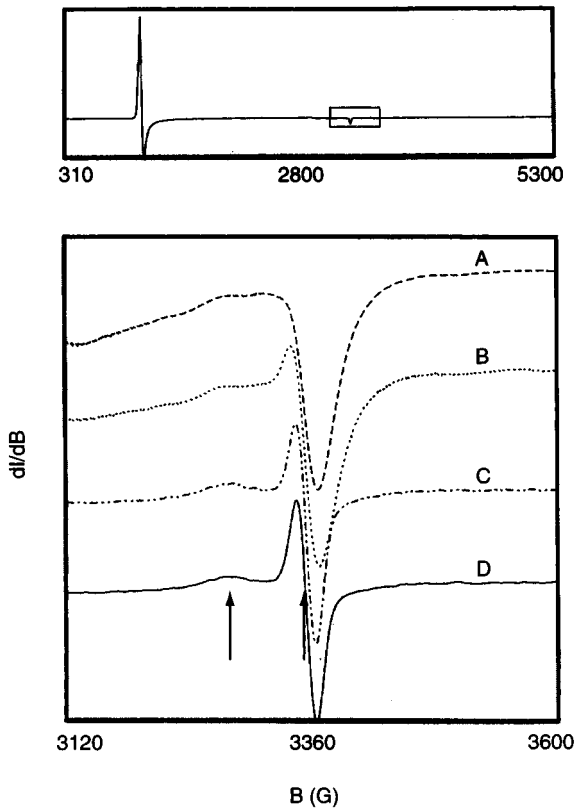

FIGURE 1: Evolution and growth of superoxide radical in partially oxygenated hemoglobin (oxyhemoglobin 55.1%, methemoglobin 6.7%, and deoxyhemoglobin 38.2%) incubated for different lengths of time at 235 K: 1 min (A); 3 min (B); 7 min (C); 17 min (D). The EPR (X-band) spectra were recorded at 8 K. Each spectrum is an average of six scans. The $g_{||}$, and $g_{\perp}$ values correspond to 2.0563 and 2.0043, respectively. The inset at the top of the figure shows the EPR spectrum of the same sample in a wider range. The area choosen for following the superoxide signal growth is indicated in the form of a box on the inset.

hemoglobin sample. New features start developing in the region of the $g = 2$ (Figure 1) after incubation at 235 K. The sharp signal which grows during incubation and overlaps with the $g_{||} = 2$ signal of high spin methemoglobin and the weak signal at lower field corresponds to the g values reported for the superoxide radical ($g_{||} = 2.0565$ and $g_{\perp} = 2.0043$) (Che & Tench, 1983). When the temperature is transiently raised to 295 K, these signals disappear and the only signal still detected in this spectral region is the $g_{||} = 2$ signal of methemoglobin (Levy et al., 1991; Rifkind et al., 1994). The finding that this signal is not produced under the same incubation condition with fully oxidized hemoglobin (Figure 2A) and fully deoxygenated hemoglobin (Figure 2B) confirms that the putative superoxide signal is not associated with the oxidized hemoglobin and that its formation requires oxygen bound to the heme.

EPR spectra of isotopically enriched partially oxygenated hemoglobin establishes that the $g = 2.0043$ band attributed to superoxide actually arises from an oxygen based radical. The O-17 fine structure for ($O^{17}-O^{17}$) consists of 11 lines and for ($O^{17}-O^{16}$) consists of six lines (Ben Taarit & Lunsford, 1973). Most of these lines could not be detected because of the presence of multiple low spin and high spin methemoglobin species (Levy et al., 1990; Rifkind et al., 1994) with absorptions in the same region and the possible contribution of a residual noncorrected background. Never-

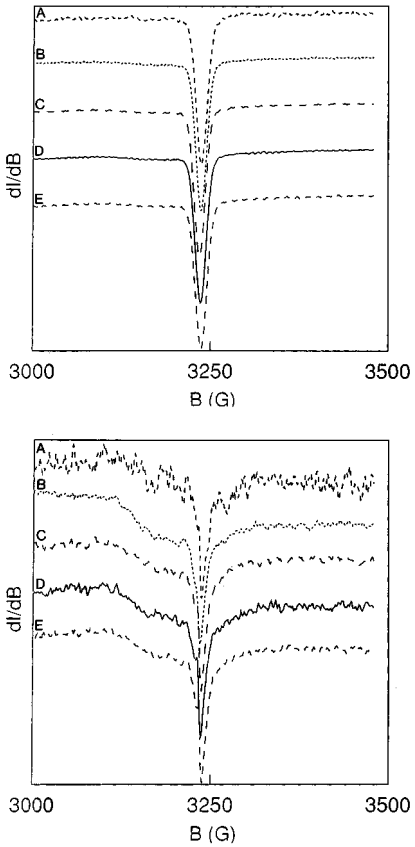

FIGURE 2: EPR spectra showing the absence of superoxide signals in 100% met hemoglobin (A, top) and 100% deoxyhemoglobin (B, bottom) recorded under conditions identical to those used for the partially oxygenated sample (Figure 1). The various incubation times are 0 min (A), 2 min (B), 6 min (C), 12 min (D), and 20 min (E). The noise level in panel B results from low methemoglobin levels.

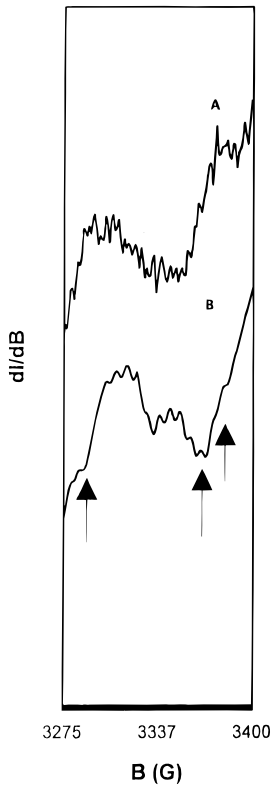

FIGURE 3: Difference EPR spectra obtained by subtracting off the $g_{||}$ signal of the methemoglobin from the superoxide $g_{\perp}$ signal of the partially oxygenated hemoglobin (after 1 min incubation at 235 K). Panel A clearly reveals the $g_3 = 1.94$ signal of the heme−peroxy-like complex. The effect of isotopic substitution (85% $^{17}O_2$) on the spectrum in this region is shown in panel B. The arrows from left to right correspond to magnetic fields at 3288, 3366, and 3382 G, respectively, and they arise from the interaction of the unpaired electron with nuclei in ($O^{17}-O^{17}$) and ($O^{17}-O^{16}$) pairs. Refer to the text for details.

theless, the effect of O-17 is clearly indicated in the relatively high field region of the spectrum. In order to improve the resolution in this region of the spectra, we have used the integrated intensity of the methemoglobin high spin signal at $g_{\perp} = 6$ to subtract off the underlying $g_{||}$ signal of the methemoglobin present in all of these partially oxygenated hemoglobin samples. Figure 3A shows the spectrum in this region for an $Hb^{16}O_2$ sample. The only signal seen is a band at $g = 1.94$. A $g = 1.94$ band has been observed in other heme proteins and iron complexes and is usually thought to be associated with a low spin Fe (III) complex (see below).

For the $^{17}O_2$ enriched sample prepared in the same way, three additional lines flank the $g = 1.94$ band. This O-17 perturbation is too great to originate from the weak $g = 1.94$ band. These new lines must therefore be associated with the superoxide $g_{\perp}$ signal with a value of 2.0043 (Che & Tench, 1983). From the position of the three additional lines in the O-17 spectrum (Figure 3B) and their location relative to the superoxide $g_{\perp}$ signal, it is possible to define the $A_{\perp}$ values for both ($O^{17}-O^{17}$) and ($O^{17}-O^{16}$). The high field shoulder at 3382 G is only 16 G away from the trough at 3366 G and 94 G from the low field shoulder at 3288 G. On the basis of literature values for the superoxide $A_{\perp}$ (Ben Taarit & Lunsford, 1973) the 16 G splitting is too small. Since the 3288 G shoulder is only 48 G from the superoxide $g_{\perp}$ at 2.0043, the $A_{\perp} = 94$ G must be associated with the ($O^{17}-O^{16}$) sextet consisting of three lines on either side of the $g_{\perp}$ signal at 2.0043. The 3366 G line which is 126 G away from the crossover for the superoxide $g_{\perp}$ then defines the ($O^{17}-O^{17}$) $A_{\perp}$ as 63 G. The appreciable difference between the two $A_{\perp}$ values (94 and 63 G, respectively) suggests an inequivalent environment for the two oxygens undergoing restricted motion within the heme pocket.

Figure 4 shows the dependence on hemoglobin oxygenation of the $g_{\perp}$ signal of the superoxide observed during low temperature incubation. These data show that the formation of this signal is specifically associated with hypoxic conditions, being much reduced at atmospheric pO_2 as well as in the absence of oxygen. Also shown in this figure is the dependence on hemoglobin oxygenation of the rate of autoxidation at ambient temperatures. A similar oxygen dependence for the autoxidation and the low temperature formation of superoxide in the heme pocket implies that the superoxide production is associated with the enhanced autoxidation of hemoglobin under hypoxic conditions. The small deviation in shape can be attributed to a minor contribution of other mechanisms of autoxidation (see below).

DISCUSSION

It has been shown that autoxidation of hemoglobin coincides with the formation of superoxide (Misra & Fridovich, 1972; Wever et al., 1973). However, the process responsible for the production of the superoxide is not fully understood.

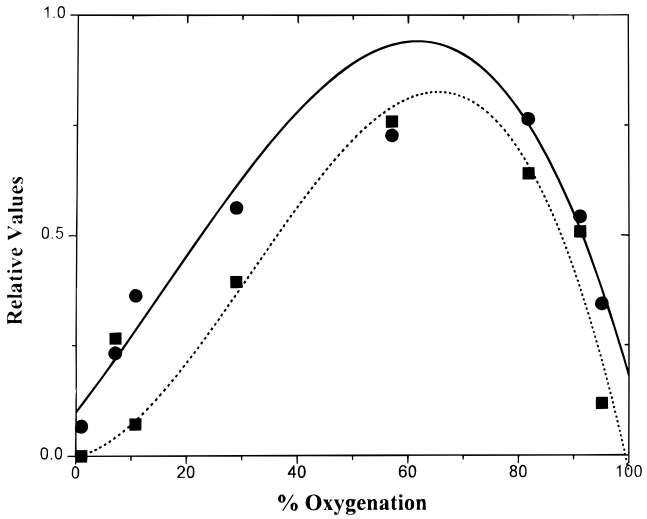

FIGURE 4: Comparison of the dependence of the rate of autoxidation (●) and superoxide signal intensity (■) on percent oxygenation. The former is obtained from visible spectrophotmetry, and the latter was obtained from EPR measurements.

On the basis of studies comparing the visible spectrum of oxyhemoglobin with low spin ferric hemoglobin (Wittenberg et al., 1974), it was suggested that in oxyhemoglobin one electron is transferred from the iron to the oxygen resulting in a complex electronically equivalent to a superoxide complex of Fe^{3+}. This contention is also consistent with the Mossbauer spectrum of oxyhemoglobin (Lang & Marshall, 1966). The transfer of an electron to oxygen producing a superoxide complex has been confirmed for Co(III) substituted hemoglobin (Hoffman & Petering, 1970). In this case Co(II) is diamagnetic and an EPR spectrum of bound superoxide is observed.

For oxyhemoglobin no EPR spectrum is observed even if it exists as an $Fe^{3+}-O_2^{\bullet-}$ complex, because of the strong coupling of the nearby unpaired electrons in the Fe^{3+} and the $O_2^{\bullet-}$. The production of observable superoxide and the autoxidation of hemoglobin from the bound oxygen, therefore, require at least a partial dissociation of the oxygen as a superoxide.

During the dissociation of molecular oxygen from hemoglobin to form deoxyhemoglobin any electron transfer that had taken place during the formation of oxyhemoglobin is reversed. Even though oxyhemoglobin may exist as a superoxide complex, it has not been previously shown that the superoxide complex can dissociate retaining its unpaired electron.

The Formation of Superoxide in the Heme Pocket. The *g* values for the free radical signal observed during 235 K incubation correspond to those previously reported for superoxide. The observation of the O-17 hyperfine structure unambiguously establishes this signal as originating from molecular oxygen and containing two inequivalent oxygens. These results confirm the assignment of this signal to superoxide radical.

Superoxide formed in biological systems has previously been observed only by either stopped-flow methods (Goldberg et al., 1979) or freeze-quenching methods (Knowles et al., 1969) which can detect the presence of transient radicals. Requirements for these fast kinetic techniques have been attributed to the rapid spontaneous dismutation of superoxide in aqueous systems. The ability to follow the formation of a relatively stable superoxide signal at low temperature during hemoglobin autoxidation (Figure 1) by conventional technique in the present case is consistent with superoxide being formed in the heme pocket. Protein fluctuations necessary to open the heme pocket are restricted at low temperature, and the superoxide dismutation requiring interaction with water is appreciably slower than its formation. As expected, this superoxide signal disappears when the temperature is raised to 295 K, a temperature at which the superoxide can leave the heme pocket and rapidly dismutate (Levy et al., 1991). However, lacking information on superoxide dismutation in the aqueous phase at 235 K, it is not possible to completely rule out the possibility of superoxide formation on the surface of the hemoglobin molecule through an outer-sphere autoxidation reaction.

The $g = 1.94$ band shown in Figure 3A is consistently observed during the early stages of the free radical formation but decreases in intensity and is frequently not detected at later stages when high levels of superoxide are still present. This behavior is consistent with that expected for an intermediate which actually precedes the formation of superoxide radicals. The exact nature of this intermediate is not known. The possibility of a complex with the superoxide still attached to the iron is particularly attractive. The $g = 1.94$ band is, however, suggestive of a low spin Fe(III)−peroxide complex which consists of bands at $g = 1.94$ as well as bands in the region of $g = 2.3$ and $g = 2.2$. The spectral region on the low field side of the $g = 2$ free radical signal from 2600 to 3100 G, where other low spin bands associated with this $g = 1.94$ signal are expected to fall, unfortunately also contain g_2 bands for the hydroxide complex, and the multiple low spin bis-histidine complexes (hemichromes) present to some extent in all methemoglobin samples (Levy et al., 1990; Rifkind et al., 1994). By comparing the EPR spectra of a number of samples, it was, however, possible to suggest that bands in the region of $g = 2.3$ and $g = 2.2$ are associated with the $g = 1.94$ band. Taken together, these three lines correspond to a heme−peroxy-like complex (Jino et al., 1991). The EPR spectral characteristics of this complex are similar to some of the intermediate states found in peroxidases (Coulson et al., 1971) and cytochrome P-450 (Tyson et al., 1972). Employing chemiluminescence experiments, Nohl and Stolze (1993) have shown that catalase insensitive heme−peroxy intermediates are involved in myoglobin oxidations with hydrogen peroxide. Others have postulated its involvement in the chemically induced autoxidation of myoglobin with H_2O_2 (French et al., 1978). Activated bleomycin formed from the oxygen complex of Fe(II)−bleomycin, or by adding hydrogen peroxide to Fe(III)−bleomycin, is also believed to be a low spin Fe(III) complex associated with a one-electron reduction of the oxygenated Fe(II) complex (Burger et al., 1981, 1983; Sam & Peisach, 1993). Although the exact nature of this putative intermediate which produces this band at $g = 1.94$ is unknown, it is almost definitely an iron complex and not an isolated free radical. This further confirms the formation of superoxide in the heme pocket from oxygen originally coordinated to the iron.

Autoxidation under Hypoxic Conditions. Contributions from both an outer-sphere bimolecular reaction of deoxygenated chains with oxygen and the dissociation or displacement of bound oxygen are thought to contribute to autoxidation (Abugo & Rifkind, 1994; Wallace et al., 1982;

Dickerson et al., 1993; Brantley et al., 1993). For myoglobin (Dickerson et al., 1993; Brantley et al., 1993) with a single subunit, the bimolecular mechanism is required to explain the enhanced oxidation at intermediate pO_2 values while contributions from the dissociation mechanism are required to explain the residual oxidation at high pO_2. For hemoglobin, a contribution from an outer-sphere bimolecular reaction was required to explain the oxygen dependence of autoxidation at low oxygen pressures where no binding of oxygen is detected spectroscopically (Levy et al., 1988). From a complete analysis of autoxidation as a function of oxygenation, it was, however, shown that a mechanism with a single rate constant for autoxidation of all non-oxygenated hemoglobin subunits cannot explain the enhanced autoxidation of hemoglobin at reduced oxygen pressures (Abugo & Rifkind, 1994). It was necessary to postulate an allosteric conformational mechanism whereby the rate of autoxidation depends on the oxygenated state of hemoglobin.

From the kinetics of autoxidation, it was not, however, possible to distinguish between an allosteric mechanism where the level of oxygenation affects the outer-sphere reaction of the deoxygenated chains or the inner-sphere displacement reaction involving the oxygenated chains. Our results on the formation of superoxide radicals during low temperature incubation strongly suggests that the enhanced autoxidation at intermediate oxygen pressures is due to an inner-sphere mechanism for which the bound oxygen is dissociated from the heme and is retained in the heme pocket. Unlike the oxygenation for which the subunit interactions cause the equilibrium constant to increase as more oxygen is bound, the maximum rate of autoxidation is found at intermediate pO_2 (Rifkind et al., 1989) where the hemoglobin is partially oxygenated. What is the nature of interactions which facilitate the dissociation or displacement of bound oxygen from partially oxygenated hemoglobin intermediates? We have previously found that distal histidine interactions are enhanced for partially liganded hemoglobin (Levy & Rifkind, 1985). Since distal histidine is the only nucleophilic side chain in the heme pocket, the possible interaction of the distal histidine with either the iron or with the oxygen bound could facilitate the displacement of the oxygen as a superoxide radical. The possibility that autoxidation proceeds through a peroxy-like intermediate (see above) raises the possibility that distal histidine interactions may facilitate autoxidation by the transfer of electron density directly to the iron−oxygen bond (Raap et al., 1978). Caughey and co-workers (Wallace & Caughey, 1975) have previously postulated such a hemoglobin oxidation process involving the transfer of electrons to oxygen by an exogenous reducing agent, which destabilized the iron oxygen bond.

Enhanced distal histidine interactions can be attributed to greater heme pocket flexibility (Levy & Rifkind, 1985) for partially oxygenated hemoglobins. Evidence that subunit interactions associated with changes in the liganded state in some of the hemoglobin chains can alter heme pocket flexibility in other chains has recently been reported (Levy et al., 1992). These results can be extended to provide a mechanism whereby the removal of oxygen from one subunit can facilitate altered heme pocket interactions in other chains.

These results provide the mechanism for enhanced production of superoxide by hemoglobin under hypoxic conditions. Within the erythrocyte the cellular detoxicants would be expected to neutralize even this enhanced oxyradical stress. However, the association of hemoglobin with the erythrocyte membrane at reduced oxygen pressures (Tsuneshige et al., 1987) provides a mechanism for the superoxide produced during hypoxic autoxidation to escape the cellular protective mechanisms and both damage the erythrocyte membrane (Rifkind & Abugo, 1994) and leak out of the erythrocyte (Rifkind et al., 1989) to damage other tissues and cells (Rifkind et al., 1992).

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