Coordination Geometry of Heme in Lactoperoxidase: pH–Dependent ^1H Relaxivity and Optical Spectral Studies

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ABSTRACT

Molar relaxivity of water proton in lactoperoxidase solution was studied as a function of pH in the range of 2–13 by spin-lattice relaxation time measurements on a Bruker AM 500 MHz nuclear magnetic resonance (NMR) spectrometer. It was shown by comparison with the molar relaxivities of met myoglobin (Mb) and horseradish peroxidase (HRP) solutions that the sixth coordination position of the heme pocket in lactoperoxidase (LPO) is vacant. Distance of the water proton in the heme pocket from ferric ion was deduced to be 2.7, 3.6 and 4.3 A for Mb, HRP, and LPO, respectively. Acid-alkaline transition for met myoglobin, horseradish peroxidase, and lactoperoxidase determined from the pH dependence of changes in the Soret absorptions were found to be characterized by pK of 8.8, 10.9, and 12.1, respectively. Proton NMR of LPO at pH = 12.2 was found to have single broad resonance considerably upfield shifted as compared to that of LPO at neutral pH. By comparison with the proton NMR of HRP and Mb at pH greater than their respective pK of acid-alkaline transition, the upfield shifted proton resonance of LPO at pH = 12.2 was assigned to be due to low-spin LPO.

INTRODUCTION

The coordination geometry of heme in native heme proteins is a problem of long standing. X-ray crystal structure studies on cytochrome-c-peroxidase (CCP) [1] and ferrimetmyoglobin (Met-Mb) [2] have established the water molecule presence in the first coordination sphere of heme iron. Because of the absence of corresponding studies on other peroxidases, it was earlier assumed that plant peroxidase such as horseradish peroxidase (HRP) also contained water bound iron heme [3, 4]. However,

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proton magnetic resonance studies have shown that the sixth coordination position of the ferric iron in HRP is vacant [5, 6]. For mammalian peroxidases such as lactoperoxidase (LPO), the nature of the sixth ligand has not yet been established with certainty. Sievers [7] has proposed that the ferric iron may be ligated by carboxylate group of the amino acid residue. However, Shiro and Morishima [8] have suggested that the sixth coordination position in LPO may either be vacant or occupied by loosely interacting water molecules. One of the methods to obtain information regarding the presence or absence of water in the first coordination sphere of ferric iron is to measure water proton relaxivity of the enzyme solution as a function of pH. Since ferric ion in LPO at physiological pH is in the high-spin state [9], it is expected that the existence of fast exchange of water molecules between bulk of the solution and the first coordination sphere of the iron should result in a large enhancement of proton-relaxation rates of the water. This method has previously been used to determine the coordination of water to the ferric ion in various heme proteins such as Met-Mb, HRP, and CCP [6, 10, 11]. Using this method, we show in the present study that LPO does not contain bound water at the heme sixth coordination position. We have also determined the distance of water proton in the heme crevice from the iron center of LPO.

Electronic absorption spectrum, spin state, and redox properties of the enzyme undergo change at acid-alkaline transition. For example, in Met-Mb and Met-Hb, kinetic studies have indicated that acid-alkaline transition is attributable to the proton dissociation of an iron bound water molecule and subsequent formation of low-spin ferric hydroxide species [12]. In case of HRP, acid-alkaline transition and spin state change characterized by pK = 10.8 cannot, however, be attributed to simple protolytic reaction of iron-bound water as HRP does not contain water coordinated to ferric ion [5]. In the case of LPO, the acid-alkaline transition has not yet been studied. Proton NMR studies [8] and kinetic studies [13] have suggested that this transition is expected to lie beyond pH = 12. Therefore, we have deduced the pK of this transition in LPO by pH dependent changes in the optical absorption at 412 nm in the pH range of 11–13. The proton NMR spectra of alkaline LPO (pH = 12.2) gives new peak at 21.5 ppm corresponding to ferric low-spin form. The nature of amino acid binding to iron center at the sixth position because of the alkaline ionization is also discussed.

MATERIALS AND METHODS

LPO was isolated from unskimmed, unpasturized cow's milk by essentially the same procedure as previously described [14–16]. Mb and HRP were obtained from Sigma Chemicals. HRP was purified by CM-cellulose and DEAE column chromatography [17]. Concentrations of the enzymes were determined spectrophotometrically by using molar extinction coefficient of 1.66×10^5 cm^{-1} M^{-1} at 409 nm for Mb [18], 1.02×10^5 cm^{-1} M^{-1} at 403 nm for HRP [19], and 1.12×10^5 cm^{-1} M^{-1} at 412 nm for LPO [20]. pH of the solution was changed by addition of μL aliquots of 1M NaOH/NaOD stock solution to the enzyme solution. The new pH was directly measured in the cuvette or NMR sample tube by Aldrich extra-long combination electrode.

Acid-alkaline transition in peroxidases can be described by:

$$P - H^+ \text{ (high spin)} \rightarrow P \text{ (Low spin)} + H^+$$

The total absorbance of solution at any pH is the sum of absorbance caused by $P-H^+$

and P species. To obtain the pK of alkaline transition, the following expression was used [21]:

$$\Delta A = [P]_0 \Delta\epsilon - [H^+]\Delta A/K \tag{1}$$

where $K = [P][H^+]/[P - H^+]$; $\Delta A = A - A_0$; A_0 is absorbance of the solution when all the enzyme is in $P - H^+$ form; A is the observed absorbance at given pH; $\Delta\epsilon = \epsilon_P - \epsilon_{P-H^+}$ and $[P]_0$ is the total enzyme concentration. The validity of Eq. 1 was confirmed by straight line obtained from a plot of ΔA vs. $[H^+]\Delta A$ (Fig. 1). Values of pK were determined for Mb, HRP, and LPO from the slope of these plots.

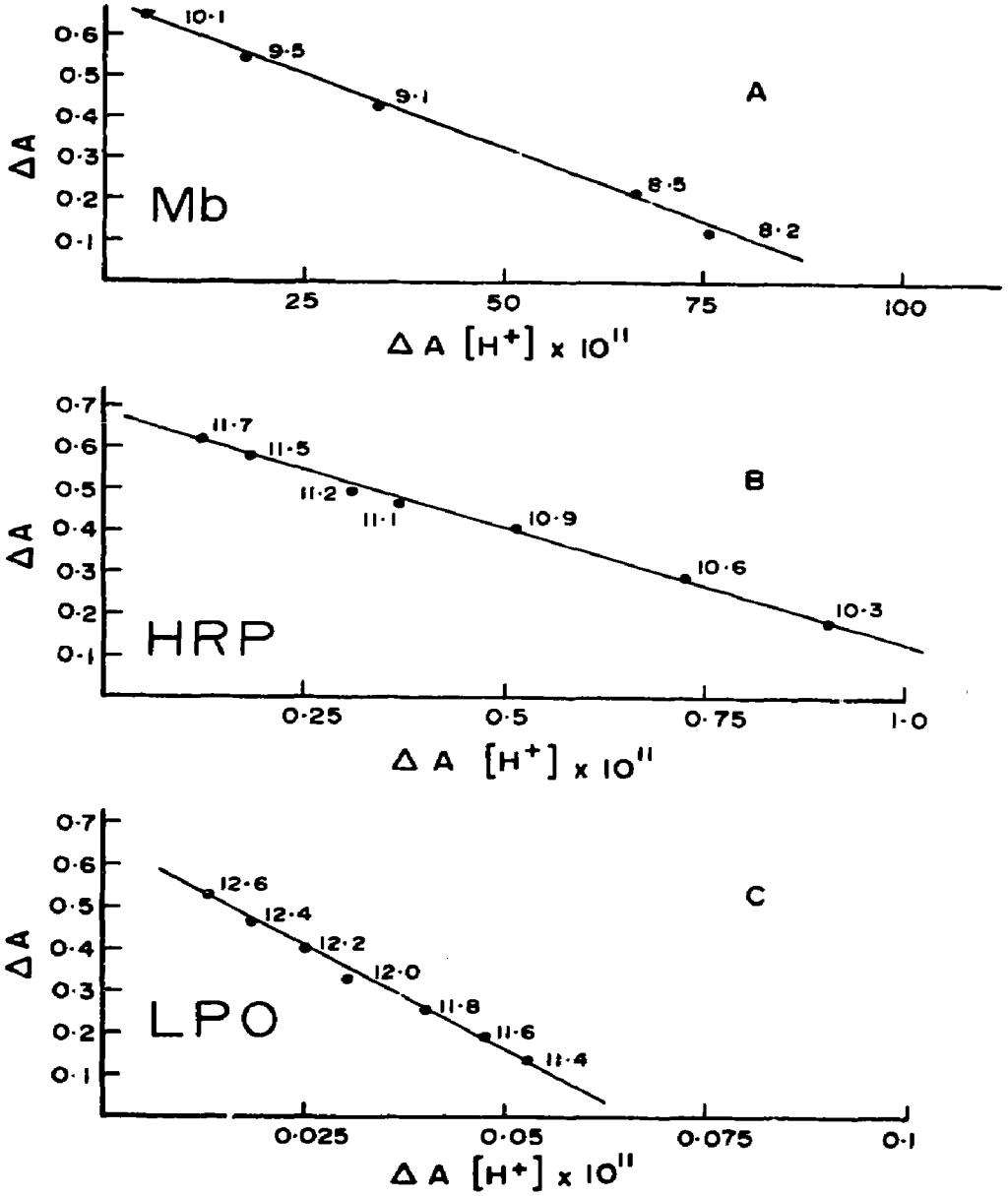

FIGURE 1. Plot of ΔA vs. $\Delta A[H^+]$ for (A) Met-Mb, (B) HRP, and (C) LPO at 23°C. The pH for each experiment are indicated in the figure.

Proton NMR measurements were carried out on a Bruker AM 500 MHz FT NMR spectrometer at 23°C. The samples were lyophilized directly inside NMR tubes with excess of D_2O. Proton chemical shifts are referred to proton signal of trace of $H_2O(d_1)$ as secondary reference at 4.75 ppm. Quoted pH are meter readings, uncorrected for isotope effects. For relaxation rate measurements, HRP and LPO were treated with Chelex-100 (BioRad) to remove any traces of free metal ions [22], and samples were prepared in deionized double distilled water. Longitudinal relaxation times (T_1) were measured at 500 MHz using $180° - \tau - 90°$ pulse.

The longitudinal (T_{1m}) and transverse (T_{2m}) paramagnetic relaxation times of bound substrate resonances can be represented by following equations [23, 24]:

$$\frac{1}{T_{1m}} = \frac{2\gamma_I^2 g^2 S(S+1)\beta^2}{15r^6}\left[\frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{7\tau_c}{1+\omega_S^2\tau_c^2}\right] \tag{2}$$

$$\frac{1}{T_{2m}} = \frac{\gamma_I^2 g^2 S(S+1)\beta^2}{15r^6}\left[4\tau_c + \frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{13\tau_c}{1+\omega_S^2\tau_c^2}\right] \tag{3}$$

where γ_I is the nuclear gyromagnetic ratio, g is the isotropic splitting factor, β is the Bohr magneton, S is the total spin of the ground state of the paramagnetic ion, r is the distance of the metal-bound substrate (observed nucleus) from the heme iron, ω_I and ω_S are the nuclear and electronic Larmor precession frequencies, respectively. In Eqs. 2 and 3, we have only included terms arising out of dipole-dipole interaction between electron spin S and nuclear spin I, which is characterized by a correlation time τ_c that modulates this interaction. The distance of water proton from iron center of enzyme can be obtained from paramagnetic relaxation rate measurements of proton nucleus of bound water using Eq. 2, provided value of auto correlation time τ_c is known. τ_c was calculated from ratio of T_{2m}/T_{1m} as described earlier [15, 16, 25]. For Fe^{+3} ($S = 5/2$) the metal-water proton distance is given by Eq. 4 [16]:

$$r(cms) = \left\{2.88 \times 10^{-31} T_{1m}\left(\frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{7\tau_c}{1+\omega_S^2\tau_c^2}\right)\right\}^{1/6} \tag{4}$$

RESULTS

ΔA was measured at different pH for Mb, HRP, and LPO; the results are shown in Figure 1. Values of pK for Mb and HRP were calculated to be 8.8 and 10.9, respectively, which agree very well with previously reported values of pK = 8.8 and 10.8, obtained from the proton NMR heme methyl shifts at different pH [5, 12]. The value of pK for LPO was similarly calculated to be 12.1 from the slope of the straight line in Figure 1c. This value is consistent with earlier suggestion from proton NMR [8] and kinetic studies of binding of benzhydroxamic acid to LPO [13] that the pK should be larger than 12.

Figure 2 shows proton NMR traces of Mb, HRP, and LPO at neutral and high pH values (above their respective pK values). Mb at pH = 6.7 has characteristic proton NMR spectrum with four heme ring methyl signals at 53.0, 72.8, 86.5, and 92.9 ppm (Fig. 2A). These peaks are characteristic of acid metmyoglobin in ferric high-spin form and agree with earlier reported proton NMR of met-Mb [5, 26]. When the pH of metmyoglobin were raised, all signals shifted up field, and four methyl

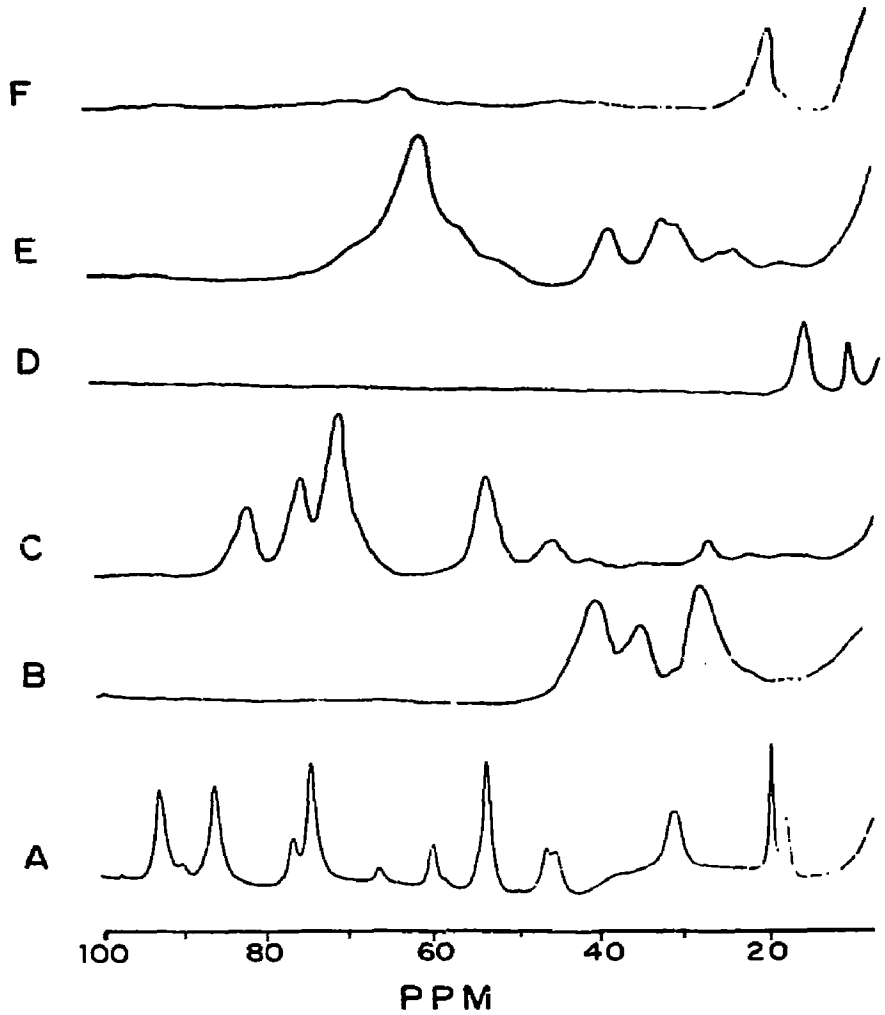

FIGURE 2. Proton hyperfine shifted NMR spectra of (A) Mb at pH = 6.7, (B) Mb at pH = 11, (C) HRP at pH = 6.8, (D) HRP at pH = 11.4, (E) LPO at pH = 7.0, and (F) LPO at pH = 12.2. Only the downfield region is shown.

peaks were located between 25 and 40 ppm above pH = 11 (Fig. 2B), characteristic of low-spin alkaline metmyoglobin [5]. HRP at pH = 6.8 has characteristic proton NMR for high-spin form with four heme ring methyl signals at 54.6, 71.3, 75.0, and 82.0 (Fig. 2C). This spectrum agrees with spectrum of native HRP reported earlier [5, 27, 28]. This spectrum of HRP was almost pH independent between 7 and 10; it changed progressively from ferric high-spin spectrum to the ferric low-spin spectrum as pH was increased above 10.8. Alkaline HRP has new peaks at 18.1 and 11.2 ppm at pH = 11.4 (Fig. 2D). LPO at pH = 7.0 has proton NMR characteristic of high-spin ferric hemes. The poorly resolved proton peaks, probably arising from the heme peripheral groups, are observed at 61.5, 55.7, 50.7, 38.3, and 30.6 ppm (Fig. 2E). The peak around 61.5 ppm has not yet been assigned with certainty because of difficulty in heme reconstitution. However, from comparison with ^{1}H NMR of HRP or Mb, the peak has been tentatively assigned to unresolved heme peripheral methyl proton resonances [29]. NMR spectra of LPO did not change significantly between pH 7 and 11.8. Figure 2F shows that alkaline LPO (pH = 12.2) has a new peak at 21.5

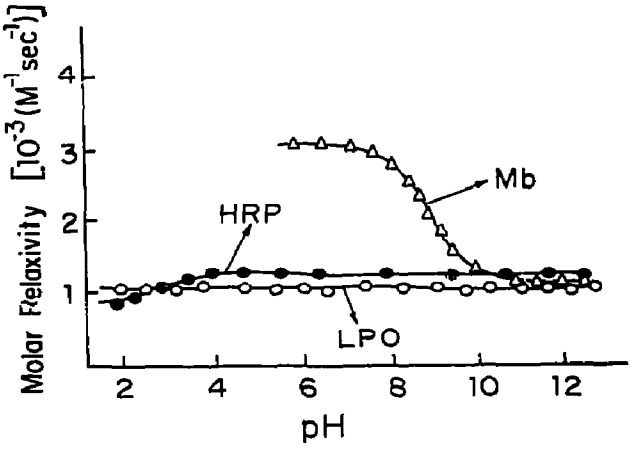

FIGURE 3. Molar relaxivity as a function of pH for Met-Mb, HRP, and LPO.

ppm, and that a weak peak of native high-spin enzyme at 61.5 ppm persists even at pH > 12.2. This may be due to a minor component of LPO having alkaline transition much above pH = 12.2. Low-spin cyanide also shows unusual proton NMR spectrum in alkaline pH region (9.6–11.2), which has been interpreted as arising because of the presence of minor components of LPO [14].

Molar relaxivities of water in LPO at different pH are plotted in Figure 3 and are compared with HRP and Met-Mb. It can be seen from the plot that molar relaxivity of neutral ferric LPO at pH = 7.0 is distinctly lower than that of neutral metmyoglobin, which has one water molecule bound to the high-spin iron, and that it is slightly lower to that of ferric HRP. T_1^{-1} value in solution of LPO is very close to that of low-spin cyanide-LPO complex, which is taken as diamagnetic contribution of ferric LPO to the relaxation [10]. If a water molecule were coordinated to the heme iron, an increase in molar relaxivity is expected on decreasing pH, as is observed in case of Mb, where relaxivity rapidly changes around alkaline transition with pK = 8.8 (Fig. 3). The fact that molar relaxivity is practically constant for LPO over the whole pH range investigated here (pH = 2–13) demonstrates that LPO like HRP has no water coordinated to heme iron. The initial small change in molar relaxivity in case of HRP between pH = 2–4 (Fig. 3) has been attributed to splitting of the heme from the apoenzyme [6]. Absence of such change in LPO in the same pH range is consistent with the recent report that heme in LPO is covalently linked to apoenzyme by disulphide bond [30].

Absence of water from first coordination sphere of iron in LPO was further confirmed by calculation of distance between water proton in the heme crevice and iron center. Presence of fast exchange limit ($T_{1m} \gg \tau_m$) was shown by temperature dependent T_{1m} measurements (data not shown). On increasing the temperature, increase in T_{1m} value was observed. Activation energy was calculated from the slope of the plot of $\log(1/T_{1b})$ vs. $1/T$ and was found to be 3.0 Kcal/mol in case of LPO. Low value of activation energy is suggested to indicate that T_{1m}^{-1} is independent of τ_m process [31, 32]. These results indicate the existence of fast exchange [15, 16, 31, 32]. The value of τ_c calculated in the same manner as described earlier [15, 16, 25, 32] from the ratio of T_{2m} to T_{1m} was found to be 4.5×10^{-10} sec at 23°C for neutral LPO, which agrees well with the reported value of 5.5×10^{-10} sec [8]. Distances of water from the iron center were calculated for Mb, HRP, and LPO using Eq. 4 (Table 1). The distance between water proton and heme iron in Mb obtained by this method is in good agreement with distance obtained from x-ray crystal structure of Met-myoglobin

TABLE 1. Distance of Water Proton from Iron Center in Case of Mb, HRP, and LPO

Heme Protein	τ_c(sec)	$r(A)$
Mb	5.0×10^{-11}	2.7(2.67)[a]
HRP	9.5×10^{-11}	3.6
LPO	4.5×10^{-10}	4.3

[a] Value in parenthesis is deduced from x-ray crystal structure (2).

(Table 1). In the case of LPO, distance is too large to be attributed to water-bound heme iron.

DISCUSSION

The NMR measurements reported in this article establish clearly that the water molecule is not bound to the iron of ferric LPO. Therefore, the acid-alkaline transition at pH = 12.1 cannot be associated with simple protolytic reaction of the iron-bound water. pH dependence of proton NMR studies [5] and pH jump kinetic [33] studies on HRP have indicated that the acid-alkaline transition with pK = 10.8 is attributable to the ionization of a protein side-chain in the distal side of heme crevice with concomitant conformational changes in the heme vicinity and ligation of the ionized amino acid residue. Probably the histidyl imidazole occupies the sixth coordination position to form a ferric low-spin form in the alkaline region [5, 34, 35]. The unusually high acid-alkaline transition with pK = 12.1 in LPO may also be related to the ionization of amino acid residue in the distal pocket of heme crevice. The exact nature of the distal amino acid residue that may be involved in ionization is not known. Kinetic study of cyanide binding to LPO [36], recent proton NMR study on LPO [8], and NMR study of binding of I^-, SCN^-, and aromatic donor molecules to LPO [15, 16, 29] have shown the existence of histidyl amino acid residue near heme iron of LPO. Therefore, the acid-alkaline transition may involve binding of this amino acid residue to ferric heme iron in alkaline LPO above pH = 12.1. It may be noted that Lukat et al. [9] have observed distinct low-spin EPR signals at elevated pH values in tris acetate buffer. Possibility of six coordinated heme formation with histidine as sixth ligand caused by denaturation of protein at elevated pH has been invoked. The proton NMR spectra of the hyperfine shifted resonances of LPO changed drastically from the ferric high-spin spectrum (pH = 7.0) to ferric low-spin spectrum (pH = 12.2) (Fig. 2F). The small hyperfine shift of the heme ring methyl peaks of alkaline LPO (pH = 12.2) also suggests fairly strong field ligand such as histidyl imidazole occupies the sixth coordination position of the heme iron of LPO. However, lysyl or tyrosyl residues with unusually high pK values are also the possibilities to consider [33].

The calculated distance of water proton from the iron center in the case of LPO is larger than that in HRP (Table 1), which is consistent with our earlier observation that the heme in LPO is more deeply buried than HRP in the heme crevice [16].

Our results establish, therefore, that the coordination geometry of heme in LPO is essentially the same as that of HRP. The common structural features at the heme sixth site in various peroxidases may be related to their fundamental functions, in

which compound I formation requires the formation of an "inner-sphere" complex between peroxide and heme iron [37]. It seems reasonable that the vacant position at the sixth position is favorable for binding of peroxides to the heme iron. This is consistent with very fast reaction rate of LPO and HRP with H_2O_2 of order of 10^7 M^{-1} sec^{-1} [38], as compared to myoglobin which is of order of 10^3 M^{-1} sec^{-1} [39].

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