Pulse Radiolysis Reduction of Myoglobin. Hydrated Electrons Diffusion Inside the Protein Matrix

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Abstract: Pulse radiolysis studies have demonstrated that the kinetics of myoglobin reduction changes with the pH of the solution. The reduction rate constant of the protein decreases with increasing pH. The net charge of the macromolecule was longtime considered to be responsible for this dependence. However, for every protein molecule bearing many reduction sites, the reduction rate of the protein would be the summation of the individual reactions of hydrated electrons on each particular reducible site. The two schemes of protein reduction were checked by the investigation of the behavior of the experimental reduction constant values (i) versus the protein charge (Brönsted approach) and (ii) versus the reducible imidazolium groups number of the protein. Although the two plots are linear and seem to assert that the two approaches are equivalent, a critical analysis shows that the Brönsted formulation cannot be applied to proteins. This scheme gives also erroneous different radii for the same protein. Thus, this work rationalizes the pH-dependent rate of reduction by an interaction of the hydrated electron with protonated histidine residues of the protein.

Introduction

It has been believed for many years that the hydrated electrons (e^-_{aq}) reacted only with the Fe(III) ion of hemeproteins.[1] It is now known that the electron-attracting site of hemeproteins is mainly the porphyrin group.[2] So, the reduction rate constant is very little affected by the oxidation state of the iron or by the presence or not of the metallic atom.[3]

Pulse radiolysis studies have also shown that the reduction rate constant of hemeproteins depends on the pH of the solution: the rate constant decreases with increasing pH.[4] Two main models have been proposed to account for this pH-behavior of the protein reduction rate constant. For the first, the electrical attraction between the e^-_{aq} and the net charge borne by the protein would act on the speed at which they come together (Brönsted−Christiansen−Scatchard relationship).[5] In this case, the electron disappears at the protein surface and the value of the reduction rate constant is ruled by the net electric charge of the protein and by the encounter distance (summation of the radii of reacting species). For the second model, the protein reduction would be due to the reaction of e^-_{aq} on the reducible sites of the protein. In this case, the measured rate constant value should be the sum of the individual rate constants of the protein reduction sites, heme included. As the reduction rate constant of amino acids is highly dependent on their side chain ionization state, this rate constant should depend on the pK value of each reacting residue. In the past, no correlation has been

found between the experimental reaction rate constants and those calculated with this model.[6,7] Unfortunately, the rate constants were calculated using pK values of free amino acids in solution. Now, from NMR titration data, it is established that the side-chain ionization of the residues depends greatly on their location inside the protein bulk.[8,9]

So, to settle between the two models, we have reinvestigated the pH-dependence of the rate constant value for horse myoglobin reduction by e^-_{aq}. This study was carried out on apomyoglobin and holomyoglobin (azidometmyoglobin) between pH 6 and 9. To have correct treatments of experimental data, the titration curve of these proteins was performed within the same pH range.

Experimental Section

Protein and Imidazolium Solutions. Lyophilized horse heart metmyoglobin (Sigma) was dissolved in pure water (protein concentration *ca.* 0.5 mM), dialyzed against water, and then dialyzed against buffered solutions containing sodium azide (15 mM) to convert the protein to azidometmyoglobin (MbN$_3$). By this procedure, the protein was N$_3$ liganded at least at 99%.[10] The deuterated azidometmyoglobin samples were prepared by dissolving the lyophilized metmyoglobin in deuterated buffer. Exact concentration of each MbN$_3$ sample was determined by visible absorption ($\epsilon_{540nm} = 11\,200$ M^{-1} cm^{-1}).[11]

Apomyoglobin (apoMb) was prepared according to the method of Rossi−Fanelli *et al.*[12] The concentration of each apoMb sample, determined by ultraviolet absorption ($\epsilon_{280nm} = 13\,500$ M^{-1} cm^{-1})[13] was between 0.3 and 0.5 mM.

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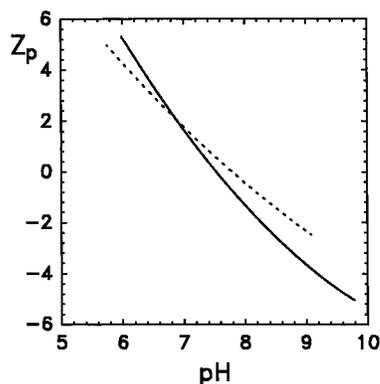
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Figure 1. Acid−base titration curves of apomyoglobin (- -) and azidometmyoglobin (—). The protein charge number $Z_p = 0$ was chosen at the isoionic point (pI = 7.8 for apoMb and 7.5 for MbN$_3$). Experimental errors: $\Delta Z = \pm 0.02$.

The pH or pD of samples was obtained by the dialysis against buffers (potassium phosphate, bis-Tris, Tris, and borate) at low ionic strength, 2.6×10^{-4} mol/L for apomyoglobin solution and 1.6×10^{-2} mol/L for azidometmyoglobin solution.

Stock imidazolium solutions (in light and heavy water) were prepared by dissolving imidazole (Sigma) in 5 mM acetic acid to obtain an imidazolium concentration at 2 mM. This stock solution was diluted 2-, 4-, and 8-fold.

For the experiments in D$_2$O, the pD values were determined from the relation[14] pD = pH$_{reading}$ + 0.4

H$^+$ Potentiometric Experiments. The protein isoionic pH was determined by measuring the pH of the protein solution after passage of a deaerated protein solution through a deionizing column (AG501-X8 Bio-Rad). The isoionic pH (7.8 for apoMb and 7.5 for MbN$_3$) were chosen as the origin points for the protein charge ($Z_p = 0$). Potentiometric H$^+$ ion titrations were performed in aqueous solution and under a nitrogen flux to avoid an acidic contamination by CO$_2$. To allow the complete proton equilibration, the titrations of apoMb and MbN$_3$ were recorded 48 h after the mixture of a known volume of titrating solutions (NaOH or HCl at 50 mM) to the protein (0.4 mM).

For apoMb, the total amount of exchanged protons, between the protein and the solvent, is directly equal to the amount of added titrating reagent. For MbN$_3$, the amount of exchanged protons was obtained by using a pK value of 4.6 for the azide ionization.[15] The number (ΔH^+) of proton equivalents by mole of protein was calculated using

$$\Delta H^+ \times [\text{MbN}_3] = [R] - \frac{10^{(4.6-\text{pH})} \times ([\text{N}_3] - [\text{MbN}_3])}{1 + 10^{(4.6-\text{pH})}} \quad (1)$$

where [R], [N$_3$], and [MbN$_3$] are the concentrations of added titrating reagent (HCl or NaOH), of total azide (15 mM) and of MbN$_3$, respectively. Above pH 7, the fraction value becomes negligible. So, in all cases, the net charge of the protein molecule, Z_p, is directly given by the number of exchanged protons ΔH^+.

The number of the imidazolium groups per mole of protein n_{Im^+} as a function of pH has been calculated using

$$n_{\text{Im}^+} = n_{\text{Im}^+(pI)} + Z_p - (n_{\text{NH}_3^+} - n_{\text{NH}_3^+(pI)})$$

where $n_{\text{Im}^+(pI)}$ and $n_{\text{NH}_3^+(pI)}$ are the numbers of imidazolium and α-amino groups at the isoionic point; Z_p and $n_{\text{NH}_3^+}$ represent the protein charge number and the amount of α-amino group at each pH value, respectively. Z_p is obtained from the titration curves (Figure 1) and n_{Im^+} for each pH value is calculated using an α-amino group pK value of 8.0.[16] At the isoionic point (protein charge zero), the number of the imidazolium groups has been calculated by considering that, at this pH, all the other residues are ionized (except for the tyrosine residue). At this isoionic point, the number of imidazolium is evaluated to be

0.39 and 0.24 for apoMb and MbN$_3$, respectively. The titration of MbN$_3$ in deuterated water was also performed, and in this case the isoionic point is 7.4.

Pulse Radiolysis Experiments. The reduction reactions were studied, around 15 °C, under pseudo-first-order conditions with about 50-fold excess of protein over initial e$^-_{aq}$ concentration (3−6 μM). In all experiments, 0.1 M *tert*-butyl alcohol was added to scavenge all the hydroxyl radicals and most of the hydrogen atoms leaving only e$^-_{aq}$ as reactive species. Deaerated solutions were obtained by using a special glassware which allows a high purity Ar gas saturation and a smooth stirring of the solution (deaeration time of 4 h).

The accelerator was a modified Febetron 707 delivering 1.8 MeV in a 7 ns (half-width) pulse.[17] The irradiation cell is in quartz (suprasil quality) with an optical path of 25 mm. Concentration of e$^-_{aq}$ was monitored at 700 nm. The decay of the e$^-_{aq}$ concentration as a function of time was measured by a fast spectrophotometric detection system with a time resolution better than 10 ns.[18] This decay allows one to obtain the rate constant for disappearance of the e$^-_{aq}$. All reduction rate constant values of free imidazolium and proteins have been corrected for the reactivity of the buffers and then normalized on their concentrations.

Results

Protein Acid−Base Titration. Figure 1 shows the charge profile of apomyoglobin and azidometmyoglobin as a function of pH. The charge variation between pH 6 and 9 is 6.6 (from +4.3 to −2.3) for apoMb and 8.1 (from +4.5 to −3.6) for MbN$_3$. This result confirms that the protein charge depends on the presence of the heme group.[9,19] Within this pH range, the titration curves are attributable to the titrations of α-amino[16] and imidazolium groups,[8,9] the imidazolium titration being preponderant. The analysis of these curves shows that the 11 histidine residues are not titrated in this pH range.[8,9,19] Moreover, the lack of sharp transition of these titration curves means that each imidazolium group gets peculiar acido-basic properties (pK value), and the pK of these residues widespreads and overlaps in this pH range. That is consistent with the NMR titration data (Table 1).

Protein Reduction by e$^-_{aq}$. For both apoMb and MbN$_3$, the reduction rate constant value decreases when pH increases and reaches a plateau around pH 8.5. The experimental reduction rate constants of apoMb and MbN$_3$ by e$^-_{aq}$ as a function of pH are shown Figure 2. The curves exhibit two distinct regions; from pH 6−7, we observe an important variation of the rate constant with pH and above pH 7 a lower one. In all cases, the reduction rate constant of MbN$_3$ is higher than that of apoMb. At isoionic pH, the reduction rate constant values of MbN$_3$ and apoMb are $1.5 \pm 0.2 \times 10^{10}$ and $0.33 \pm 0.06 \times 10^{10}$ M^{-1} s^{-1}, respectively. Thus, at a protein net charge of zero, the difference of reduction rate constant values of MbN$_3$ and apoMb is about 1.2×10^{10} M^{-1} s^{-1}. At the plateau value, the difference is *ca.* 1.0×10^{10} M^{-1} s^{-1}.

First Hypothesis. *The net charge of the protein molecule only acts on the kinetic of the e$^-_{aq}$ disparition.* In this case, the Brönsted formulation describes the encounter rate between the two charged species (protein and e$^-_{aq}$). So, the values of the experimental reaction rate constants (k_{exp}) must be corrected for the effect of the ionic strength of the solution. The

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Table 1. pK Values for Histidine Residues in Apomyoglobin and Azidometmyoglobin[a]

	24	36	48	81	82	113	116	119	H-2	H-3	H-4
ApoMb	6.15	7.83	5.30	6.45	<4.8	<5.9	6.78	6.25	6.05	5.6	<5.7
MbN$_3$	6.08	7.87	5.94	6.92		6.67	7.03	7.18			

[a] Data obtained from NMR studies.[9] We use the notations given by Cocco *et al.*

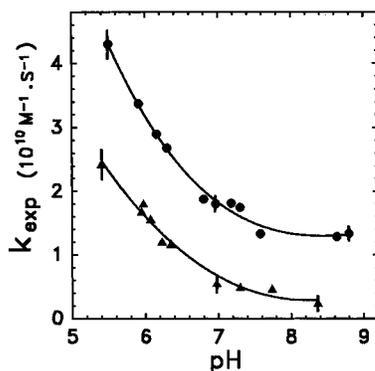**Figure 2.** Experimental values of the reduction rate constant (k_{exp}) of apomyoglobin (▲) and azidometmyoglobin (●), by hydrated electrons, as a function of pH. Error bars are given at 3 pH. The bars reveal the extreme experimental errors.

Brönsted−Bjerrum equation[20,21] gives

$$\log(k_{exp}) = \log(k_{\mu=0}) + 2 \times B \times Z_p \times Z_e \times \mu^{1/2} \times$$
$$(1 + d \times g \times \mu^{1/2})^{-1}$$

where $k_{\mu=0}$ is the rate constant for a medium of ionic strength $\mu = 0$; Z_p and Z_e are the charge number of the reacting species (p for protein and e for hydrated electron); μ is the ionic strength of the experimental medium; d is the distance of the closest approach of the electron to the protein. B and g are two parameters function of the dielectric constant ϵ and the absolute temperature T

$$B = 1.824 \times 10^6 \times (\epsilon \times T)^{-3/2}$$

$$g = 50.29 \times (\epsilon \times T)^{-1/2}$$

the dielectric constant value (82) being that determined for water at 15 °C, temperature of the radiolysis experiments.

The Christiansen−Scatchard equation[22,23] gives the dependence of the rate constant on the coulombic interactions

$$\log(k_{\mu=0}) = \log(k_0) + Z_p \times e^2 \times (2.3 \times \epsilon \times (r_e + r_p) \times$$
$$k_B \times T)^{-1}$$

in which k_0 represents the rate constant in the absence of coulombic interaction *i.e.*, in a medium of infinite dielectric constant; e is the electron charge (4.8×10^{-10} cgs units); $r_e + r_p$ is the encounter distance (sum of the radii of the hydrated electron r_e and of the protein r_p); k_B is the Boltzmann constant (1.38×10^{-16} erg deg^{-1}). The charge number of the protein, Z_p, at each pH is given by the acid−base titration curve. Plots of the decimal logarithmic value of protein reduction rate constants extrapolated at $\mu = 0$ (log $k_{\mu=0}$) versus the charge number of the protein (Z_p) allow us to determine the radius of the macromolecule. For MbN$_3$ and apoMb, the plots $\log(k_{\mu=0})$ = f(Z_p) are presented Figure 3. From the slope of these plots
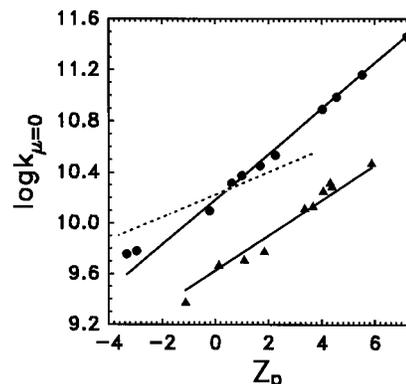

Figure 3. Brönsted plots for the reduction kinetics of (▲) apomyoglobin, (●) azidometmyoglobin and (- -) metmyoglobin: $k_{\mu=0}$, reduction rate constant extrapolated at $\mu = 0$, as a function of Z_p, protein charge number. The slope α of the curves should be inversely proportionnal to the sum of the e$^-_{aq}$ and protein radii. The slope α is determined with an error of $\Delta\alpha \leq \pm 0.012$ which gives an error $\Delta r_p \leq \pm 1.2$ Å on the values of the apomyoglobin or azidometmyoglobin radius r_p. For metmyoglobin, $\Delta r_p = \pm 4$ Å.

and using a radius of 2.7 Å for e$^-_{aq}$,[24] we determined radii (at ± 1.2 Å) of 13.5 and 19.5 Å for MbN$_3$ and apoMb, respectively. A previous paper has reported the experimental rate constant of the reduction of horse heart metmyoglobin, by e$^-_{aq}$, as a function of pH.[2] We have plotted $\log(k_{\mu=0})$ *vs* Z_p (Figure 3) by using the metmyoglobin charges determined by acid−base titration. The radius of metmyoglobin molecule is of 32 Å $\pm$ 4 Å. These radius values are different from the value of 14.5 Å, obtained by neutrons scattering.[25] The discrepancy between the various values that we obtained (13.5, 19.5, and 32 Å) suggests that this model cannot be correct.

Second Hypothesis. *For a given pH, the reactivity of some particular residues determines the reaction rate of e$^-_{aq}$ with the protein.* Within the investigated pH range, only the ionization state of the imidazole groups can vary. Moreover, the imidazole reactivity to e$^-_{aq}$ is strongly dependent on their ionization state. The reaction rate of e$^-_{aq}$ with the free imidazolium group is about 200-fold faster than that with free imidazole[7,26] and 15-fold higher than that of other amino acid side chains.[6,7] So, the increase of the experimental constant value between basic and acid pH would be due to the protonation of the imidazole group. The value of the protein reduction rate constant should be obtained by the summation of the individual rate constant value of each reducible imidazolium. The imidazolium number as a function of the pH was obtained from the charge number Z_p of the protein measured by the acid−base titrations and using the relation (given in the Experimental Section)

$$n_{Im^+} = n_{Im^+(pI)} + Z_p - (n_{NH_3^+} - n_{NH_3^+(pI)})$$

Figure 4 gives the plots of the reduction rate constant k_{exp} versus

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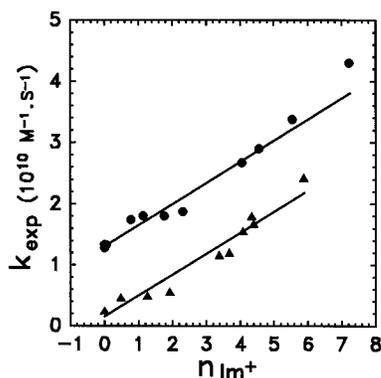(23) Scatchard, G. *Chem. Rev.* **1932**, *10*, 229−240.

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Figure 4. Experimental values of the reduction rate constant (k_{exp}) of apomyoglobin (▲) and azidometmyoglobin (●), by hydrated electrons, as a function of their number of titrated imidazolium, n_{Im^+}. The slope k of curves gives the best average value of the reduction rate constant for each imidazolium in the protein. Error on these slopes $\Delta k = \pm 0.5 \times 10^9$ M^{-1}·s^{-1}.
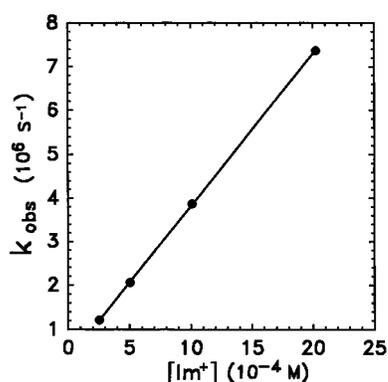

Figure 5. Reduction of deuterated free imidazolium by hydrated electrons: pseudo-first-order rate constant values (k_{obs}) as a function of the imidazolium concentration. The slope gives the rate constant value: $k = 3.4 \pm 0.1 \times 10^9$ M^{-1}·s^{-1}.

n_{Im^+} obtained for apoMb and MbN$_3$ with pH variation. Assuming that the experimental reduction constant values are only due to the number of protonated imidazole groups in the protein, the slope of these plots represents the value of the imidazolium reduction rate constant for each of these protein residues. Using a linear regression, the reduction rate constant of an imidazolium of the protein matrix is $3.4 \pm 0.5 \times 10^9$ M^{-1} s^{-1} for both apoMb and MbN$_3$. The constancy of this value, identical to that found for protonated free imidazole group in water ($3-4 \times 10^9$ M^{-1} s^{-1}) (our data and[7,26]), suggests that this second model should be correct.

Protein and Imidazolium Reduction in Heavy Water. Measurements of protein reduction in heavy water were performed at two pD values, 9.2 and 7.6. In this pD range, our titration shows that only one imidazolium group is unmasked in the deuterated protein. The variation of the reduction kinetic constant is about $3 \pm 0.5 \times 10^9$ M^{-1} s^{-1}. Thus, the reduction rate constant values of imidazolium in protein, deuterated or not, are the same. Moreover, for both free imidazolium dissolved in light water and in heavy water, the reduction rate constant value is also the same: $3.4 \pm 0.1 \times 10^9$ M^{-1} s^{-1} (see Figure 5). These same magnitudes of the rate constant indicate that the rate determining step of the reaction is not an hydrogen or deuterium atom transfer but an addition of e$^-_{aq}$ on the imidazolium group. They also show that the location of the imidazolium groups at particular sites of the protein does not affect their reduction process. This strongly suggests that, in the myoglobin molecule, the electron-imidazolium encounter only results from a random migration of the hydrated electrons.
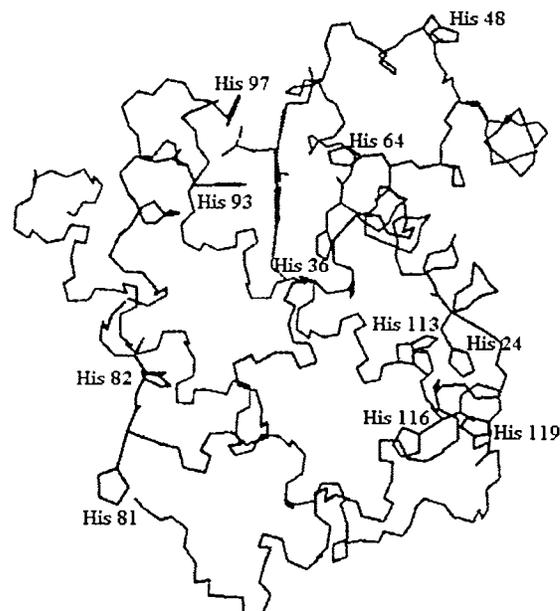

Figure 6. Repartition of the histidine residues inside the myoglobin molecule. Structural data obtained from X-ray cristallography.[30] Representation made using the WinMGM molecular graphics program.[31]

Discussion

The present study shows that the reduction rate constants of apomyoglobin, azidometmyoglobin, and metmyoglobin by hydrated electron depend on the pH value. This pH dependence of the reduction rate constant was already observed for other proteins.[2,4,6] The variation of the total net charge of these proteins as a function of pH has driven many authors to assume that the change of the rate constants could be ascribed to the electric attraction between the more or less positive-charged protein and the negative e$^-_{aq}$.[2-6] Moreover, the good linearity of the Brönsted plot using the experimental data has reinforced this interpretation. To check if the limiting step of the protein reduction by e$^-_{aq}$ is the electrostatic attraction between protein and the e$^-_{aq}$, we have performed the reduction of various derivatives of the same protein. Although our results show excellent linear fits with Brönsted relationship, we must introduce, in this relation, different radius values for the same protein. So, the electrostatic attraction cannot be used alone, to interpret the pH-modulation of the myoglobin reduction. In addition, the Brönsted law requires the restrictive condition that e$^-_{aq}$ interacts with the particle by their only own reciprocal charge. This is not the case here since, in the studied pH range, the increase of the positive charge borne by the protein is the result of the protonation of some imidazole groups which are, themselves in their charged form, highly reducible. So the k_0 value in the Brönsted relation should vary with the protein charge. This is impossible, since the value of k_0 must be strictly independent on the particle charge. For these reasons, the Brönsted relation cannot be applied to proteins and must be rejected.

The variation of the reduction rate constants as a function of pH may also be described by a change of the reactivity of some amino acid side chains upon their ionization. In this case, the value of the experimental reduction rate constant should be the summation of the rate constant value of each protein reduced group. Previous works have already attempted this approach.[6,7] Unfortunately, these works have used, in their calculations, the pK of free amino acids. Since NMR studies of myoglobin histidine residues have shown that their pK depend on their location inside the protein (see Figure 6 and Table 1 giving

histidine positions with their pK values), a strong discrepancy was found between the experimental values and those calculated. The reinvestigation of such an approach, using the correct histidine pK, gives the experimental pH-pattern of the protein reduction rate constant. The higher values of the reduction rate constant for MbN$_3$ compared to that of apoMb confirm that the heme group is also reduced by e$^-_{aq}$. As, between pH 9 and 5.5, the ionization state of the heme-azide group does not vary, the reduction constant evolves concomitantly with the change of imidazole groups in imidazolium groups. The one and only difference with apoMb is that the pK values of the same imidazole groups are slightly shifted. It is remarkable that the average value of the reduction rate constant of protonated histidine groups is identical for all myoglobin derivatives (apoMb, MbN$_3$, metmyoglobin) and close to that of imidazolium in aqueous solution. So, this study shows that the reduction kinetic of protein imidazole groups does not depend on their location but only on their protonated state. It also shows that the experimental reduction kinetic of proteins is, in the studied pH range, directly linked to the reactivity of some particular groups. In hemeproteins, the fastest reducible sites (imidazolium groups and the heme) are enough to account for the values of reduction rate constants.[7,3]

At this stage of discussion, we must remember that it has been spectrally shown that the reduction of hemeproteins by e$^-_{aq}$ leads to formation of histidine radical[3] and porphyrin anion.[2,3] Thus, the pH-pattern of the reduction rate constant of horse heart myoglobin should be ascribed to the change of the imidazole reactivity with their ionization state. Our results show that, within the investigated pH range, the limiting step for hemeprotein reduction by e$^-_{aq}$ is the electron reaction with imidazolium groups differently localized inside the macromolecule. This suggests that, after the initial encounter of e$^-_{aq}$ with the protein surface, the e$^-_{aq}$ which have not reacted with some histidine residues located on this surface would diffuse within the protein matrix and reach the reducible sites (heme or histidine). Indeed, as small molecules (O$_2$ and CO) diffusing inside protein moiety, the e$^-_{aq}$ can cross the protein and attack all reducible sites (see ref 27 and articles therein).[28] The consumption of an unequal partition of the electrons number on the heme and on the protein matrix, shown by Hasinoff, reinforces this point of view.[3] The e$^-_{aq}$ partition between the different reducible sites is essentially ruled by the relative values of their reduction kinetic constant. From these data, partial and oriented electron transfer along particular peptide chain[29,30] from the protein surface to the reducible sites (histidine and heme) cannot be expected and appears very uncertain for the myoglobin molecule. The best and simplest explanation of the observed facts is that the heterogeneous population of reducible sites inside the protein (see the histidine distribution in the myoglobin matrix, Figure 6) is statistically reached during a random migration of e$^-_{aq}$ throughout the protein interior. The rate constant would be then ruled by the reaction of these e$^-_{aq}$ on the encountered reducible groups during their random collisions inside the protein.

More interesting for our purpose is the consideration of the effect of surrounding solvent on the imidazolium group. Indeed, the reduction of these groups within the protein solubilized in aqueous buffer exhibits the same kinetic constant value as that of free histidine in water solution. In heavy water, the effects are exactly the same. Thus, in both cases, the active groups are only the imidazolium group and the solvated electron which both give the imidazolium radical.

In conclusion, the net charge of the protein cannot be responsible for the pH-pattern of the protein reduction rate constant values by e$^-_{aq}$. The kinetic constant value can be reasonably described by the summation of the reaction of e$^-_{aq}$ on each individual reducible sites more or less buried inside the protein. In fact, since histidine residues (hindered or not within the protein) are reached by the hydrated proton, there was no reason for the hydrated electron to not also reach directly the histidine residues. To generalize the interpretation that the diffusion of the hydrated electron to the buried residues at different states of protonation is the relevant parameter of the rate determining reduction of proteins, works are in progress on various proteins having a different composition of reducible amino acids.

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