On the Origin of the Non-Haemic Iron Transferrin ESR Signal: ESR Investigations on Histidine-Iron-Ascorbic Acid Systems

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ESR, Ferric High Spin Iron Complex, Ascorbic Acid

The nature of the ferric high spin iron complex located at $g = 4.3$ has been investigated by means of electron spin resonance spectroscopy. It could be shown that the iron is bound to two histidines, three ascorbic acids, and one bicarbonate. This agrees well with previous findings according to which the ligand field of iron is composed mainly of oxygen and nitrogen atoms. Another low-field signal located at $g = 9.5$ appears always concomitantly with the $g = 4.3$ signal. It should be due, therefore, to a transition between the two sublevels of the low-lying Kramers doublet in one principal direction.

Introduction

An asymmetric signal at $g = 4.3$ has been observed in many tissues [1]. Several attempts have been made to assign this signal. It seems to be indicative of ferric high spin iron atoms in a rhombic ligand field. Such a configuration can, however, exist in several iron containing compounds and has been observed e.g. in blood samples and certain other body fluids. In these cases, it has been assigned to iron-transferrin [2, 3] and lactoferrin [4], respectively. More recently, it has also been detected in oxidized putidamonooxin, an enzyme present in *Pseudomonas putida* [5].

The exact nature of the complex, that is the type of ligands, is still unknown. Since this signal appears in many biological iron-containing tissues and is changed in concentration in certain types of cancer [1], it seems to be important to determine the ligands of these high spin ferric ions. From the results obtained thus far, one might conclude that the signal arises from a transition between the two sublevels of the middle Kramers doublet. The zero-field splitting parameter D was determined to be less than 1 cm^{-1} which indicates that the ligand field is composed mainly of oxygen and nitrogen atoms.

Certain indications about the ligand field were obtained when we investigated the influence of histidine-iron complexes on the $g = 2.005$ signal of erythrocytes treated with ascorbic acid [6]. In these instances, not only a reduction in spin concentration and a change in the shape of the signal at $g = 2.005$ but also a considerable increase of the $g = 4.3$ signal could be noticed. For this reason, the influence of the histidine-iron-ascorbic acid system on the latter signal has been investigated in order to elucidate the structure of the basic unit of the iron complex being responsible for the signal observed at $g = 4.3$.

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Materials and Methods

The ESR spectra were obtained with a Varian E-9, 100-kHz modulation X-band spectrometer. The modulation amplitude was 1.25 mT and the microwave power 5 mW for all samples investigated at 77 K. The relative spin concentrations were obtained by taking the peak-to-peak heights of the signal at $g = 4.3$.

Histidine, alanine, $FeCl_3$, $FeSO_4$, and ascorbic acid were purchased from Sigma Co. Munich or Merck Co. Darmstadt resp. and were dissolved in bidistilled water in concentrations given in the Figures. Bicarbonate and imidazole were obtained from Riedel-de Haen, Seelze-Hannover. Shortly after preparation they were frozen at 77 K.

Results and Discussion

The effect of $FeCl_3$ on histidine with the resulting formation of the $g = 4.3$ signal is shown in Fig. 1. At concentrations < 100 mM, the signal is rather small while at larger concentrations the intensity increases considerably. A determination of the pH value shows that at about 100 mM of $FeCl_3$ the pH value decreases from about 7 to 3 indicating that the

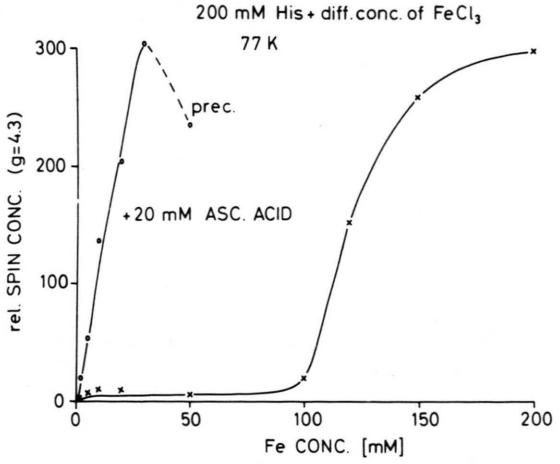

Fig. 1. The effect of histidine and ascorbic acid on the Fe^{3+} ESR signal at $g = 4.3$ measured at 77 K.

complex is in solution only for Fe concentrations > 100 mM.

The effect of pH is also shown in Fig. 3 in which case the corresponding pH was attained by adding HCl to a solution containing 200 mM His and 5.6 mM $FeCl_3$. As can be seen, the signal intensity rises sharply at a pH < 4.

When ascorbic acid (20 mM) is added to a His-Fe^{3+} solution, the signal intensity rises considerably at small Fe^{3+} concentrations (s. Fig. 1). This cannot be due to a change in pH, since the pH change is about the same with or without ascorbic acid. It might be rather caused by an interaction between histidine and ascorbic acid or its radical formed by the presence of oxygen [7]. Obviously, there is also an interaction between Fe^{3+} and ascorbic acid [6].

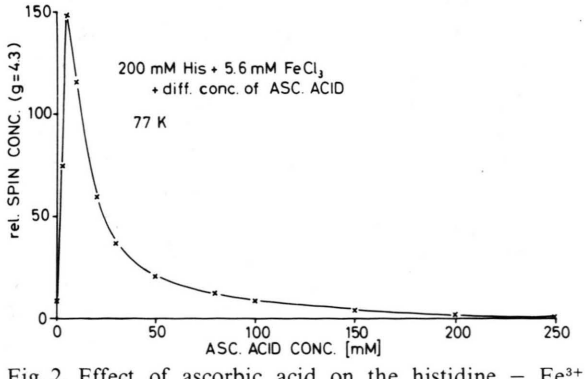

Fig. 2. Effect of ascorbic acid on the histidine – Fe^{3+} interaction as determined by the Fe^{3+} ESR signal at $g = 4.3$.

Both types of interaction might prevent the complex from precipitation which, however, occurs at Fe^{3+} concentrations > 30 mM. At this concentration the pH drops below 6. It should be pointed out that at Fe^{3+} concentrations > 150 mM, the solution clears up and the signal intensity rises again. At this concentration, the pH of the solution is < 3 and the effect observed should be similar to that shown in Fig. 3.

From the results obtained one might conclude that both ascorbic acid and pH are important for the His-Fe^{3+} interaction. For this reason, the effect of different concentrations of vitamin C on this interaction has been investigated. The influence on the signal intensity is shown in Fig. 2. As can be seen, an optimum is obtained at about equimolar concentrations of Fe and ascorbic acid, using a histidine concentration of 200 mM. When 12 mM His is used, the optimum is obtained at an Fe and ascorbic acid concentration ratio of about 1:3. In these experiments, the pH was not adjusted. It decreases gradually from about 7.2 to 5 with increasing vitamin C concentrations.

The effect of pH on the signal intensity of the ternary system histidine-iron-ascorbic acid is shown in Fig. 3. As pointed out earlier, the signal intensity

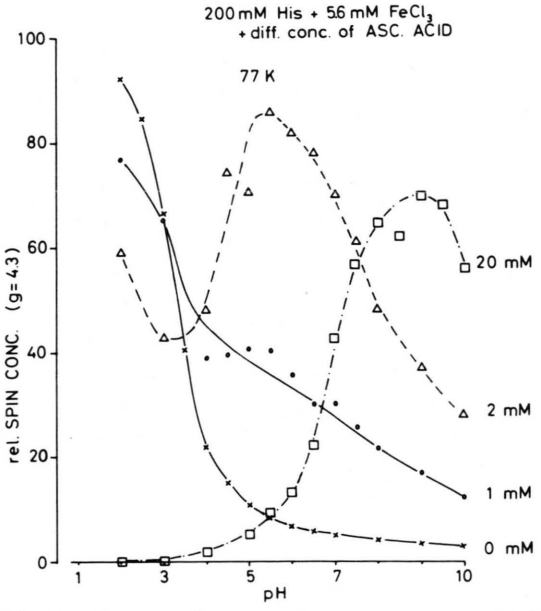

Fig. 3. Influence of pH on the ternary system histidine – Fe^{3+} – ascorbic acid as determined by the Fe^{3+} ESR signal at $g = 4.3$ and by using variable ascorbic acid concentrations.

of the histidine-Fe^{3+} system increases with decreasing pH. When ascorbic acid is added, an interesting phenomenon can be observed: at small pH values, the signal intensity decreases with increasing vitamin C concentrations. At larger pH values (> 4.5), the opposite effect occurs. In the presence of vitamin C the intensity exhibits an optimum at pH values between 5.5 and 9 depending on the vitamin C concentration. The effect seems to be very specific, since the transition in signal intensity occurs at about equimolar concentrations of Fe^{3+} and ascorbic acid. Furthermore, it can be noticed that the optimum is shifted to larger pH values with increasing ascorbic acid concentrations. The optimum remains, however, at about pH 9 for vitamin C concentrations exceeding 20 mM. It increases slightly in intensity only, if the vitamin C concentration increases (about 1.4 times if 1 M is used instead of 20 mM). The decrease in signal intensity at larger pH values is caused by a precipitation.

The deviations of the values obtained at pH 5 (2 mM curve) and pH 8.5 (20 mM curve) from the lines drawn are statistically significant. They might be caused by the pK values of histidine. Another explanation cannot be offered yet.

These results show very clearly that ascorbic acid interacts with the histidine-iron system and suggest that it might be the ligand which contributes the oxygen atoms. Previous suggestions proposed that five of the six groups to which Fe^{3+} in transferrin is bound are derived from the protein-3 tyrosines and 2 nitrogen ligands, probably from the imidazole part of histidine [8]. The present investigations suggest that ascorbic acid instead of tyrosine might be the ligand in question or might be able to replace it, at least. It should be mentioned that no signal could be observed when tyrosine was used instead of ascorbic acid.

Based on these data, a direct interaction between Fe^{3+} and vitamin C should also result in the $g = 4.3$ signal possibly at a certain pH only. ESR investigations of such a solution exhibited this signal in a small pH interval only (s. Fig. 4). The optimum occurs at pH 6.5 and is shifted to higher pH values when histidine is added. As can be seen, the optimum is at the physiological pH (7.4) if about 12 mM of histidine were added. Since this pH is predominant in living systems, it can be concluded that the concentration ratio which results in the optimum spin concentration (Fe^{3+}: ascorbic acid: His = 1:3:2) prevails in transferrin. This agrees well with the assumption suggested previously [8] that the ferric ion is bound tightly to two histidines and three tyrosines or ascorbic acids, resp.

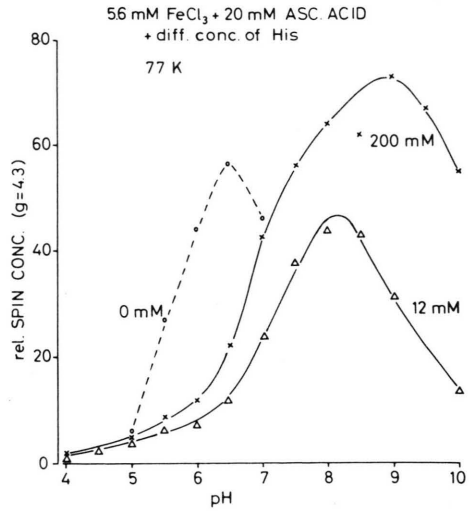

Fig. 4. Influence of pH on the ternary system histidine – Fe^{3+} – ascorbic acid as determined by the Fe^{3+} ESR signal at $g = 4.3$ and by using variable histidine concentrations.

In the experiments conducted, the pH value was obtained by adding either HCl or NaOH. In blood plasma, the appropriate pH value is obtained mainly by the bicarbonate and the protein systems. In the latter case, the imidazole ring of histidine is of special importance. This might be the reason why the usual sixth position of Fe^{3+} is probably occupied by the bicarbonate ion [8].

From these results it might be concluded that Fe^{3+} in transferrin is bound to two histidines, three ascorbic acids, and one bicarbonate.

When imidazole or alanine are used instead of histidine, the signal at $g = 4.3$ will also appear, its intensity is, however, much smaller. In the case of alanine it is about half of that obtained with imidazole indicating the importance of the ring system.

A small signal is also present if Fe^{2+} ($FeSO_4$) is used instead of Fe^{3+}. This is probably due to the partial oxidation of Fe^{2+} by the oxygen present.

Furthermore, it should be pointed out, that there is another low-field signal at $g = 9.5$ with a low intensity. It always appears concomitantly with the $g = 4.3$ signal. Therefore, it should be due to a transition between the two sublevels of the low-lying Kramers doublet in one principal direction.

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