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Binding of carboxylate anions at the non-heme Fe(II) of PS II. I. Effects on the $Q_A^-Fe^{2+}$ and Q_AFe^{3+} EPR spectra and the redox properties of the iron

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Abstract

We have examined the effects of a number of carboxylate anions on the iron-quinone complex of Photosystem II (PS II). Typical effects are the following. In the state $Q_A^-Fe^{2+}$ oxalate enhances significantly the $g = 1.84$ EPR resonance while, for example, glycolate and glyoxylate suppress it. The anions have variable effects on the iron midpoint potential. Formate and oxalate raise significantly the E_m of the iron. Glycolate lowers the E_m significantly and the E_m shows a weak pH dependence. In the presence of glycolate the native plastosemiquinone (Q_B^-/Q_BH_2 couple) can oxidise the iron. Glyoxylate also lowers the E_m, but the E_m shows a greater pH dependence than with glycolate but still weaker than the -60 mV/pH unit of the untreated iron. The Fe^{3+} EPR spectra are characterised by small but distinct shifts, while in addition an unusual resonance at close to $g = 4.3$ is observed. These as well as the temperature dependence of the spectra are analysed by a spin-Hamiltonian model. Comparison with competition studies in the companion paper indicates that the anions bind as iron ligands displacing bicarbonate.

Keywords: Photosystem II; Iron-quinone complex; EPR; Carboxylate anion

1. Introduction

The non-heme iron of Photosystem II (PS II) has a number of properties which differentiate it from its analogue in the purple-photosynthetic bacteria. Besides spectroscopic differences and its redox activity the iron of PS II can reversibly bind small molecules such as NO [1] or CN [2]. Bicarbonate-NO competition studies have suggested that bicarbonate is the natural labile ligand to the non-heme iron [3]. Normal rates of electron flow require the presence of this anion (see, e.g., Refs. [4,5]. The mode of bicarbonate binding and action, however, is not fully understood at present.

A well known class of proteins which require CO_2 for their functioning are the transferrins. In the transferrin proteins CO_2 in the form of carbonate is required as a synergistic anion for iron binding. An X-ray structure determination of lactoferrin has shown that the protein provides four ligands to the iron (two tyrosines one histidine and one aspartate) while carbonate is bound to the iron as a bidentate ligand [6]. Schlabach and Bates [7] have examined an extensive list of carboxylate anions which can substitute for carbonate as the synergistic anion in Fe-transferrin. More recently Dubach et al. [8] have reported on EPR spectra of iron-transferrin anion complexes and have provided insights into the mode of binding of these anions as well as of carbonate to the iron.

Prompted by these studies, we examined in a preliminary report [9] the effect of these anions upon their substitution of bicarbonate in Photosystem II. We elaborate here on the effects of these anions on the $Q_A^-Fe^{2+}$ and Q_AFe^{3+} EPR spectra and the redox properties of the Fe^{3+}/Fe^{2+} couple.

Abbreviations: PS II, Photosystem II; BBY, thylakoid membrane fragments isolated according to Refs. [10-12]; Q_A, primary quinone electron acceptor; Q_B, secondary quinone electron acceptor; Mes, 4-morpholinethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

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2. Materials and methods

2.1. Photosynthetic membranes and treatment with the anions

The samples used in this study were BBY or thylakoid spinach preparations isolated by standard procedures [10–12]. Chlorophyll concentration of the samples used in the EPR studies was typically 3 mg/ml.

The carboxylate anions were buffered and pH adjusted (Mes, Hepes or Tris for the respective pH ranges, 6–6.5, 7–7.5, 8–8.5) at the same pHs of the samples prior to addition. The samples were incubated in the air with the various anions (at 40 mM concentrations unless otherwise specified) at pH 6–6.3 for 1–2 h. At pHs higher than 7, longer incubation periods were required, in some cases 12–15 h.

2.2. Electron paramagnetic resonance spectroscopy

EPR measurements were obtained using a Bruker ER 200D-SRC spectrometer (9.42 GHz microwave frequency) interfaced to a personal computer and equipped with an Oxford ESR 9 cryostat. g-Values were determined with a Bruker NMR Gaussmeter and an Anritsu MF76A frequency counter. For the study of the temperature dependence of the spectra, the helium flow rate was adjusted for minimum thermal gradients and the temperature calibration was tested both before and after each set of measurements with a thermocouple placed in the position of the sample. Nevertheless, temperature uncertainties are unavoidable and these account for some deviations at low temperatures in Fig. 4.

2.3. Redox titrations

Redox potentials were adjusted by variable mixtures of ferri/ferrocyanide and/or ascorbate and measured with a Metrohm combined microelectrode (Ag/AgCl/KCl 3 M) connected to a Fluke 8840A multimeter.

2.4. Ligand field analysis of the Fe^{3+} ($S = 5/2$) EPR spectra

The ground ^{6}S term of the Fe(III) in the presence of a ligand field of axial symmetry splits into three Kramer's doublets ($M_s = \pm 1/2$, $\pm 3/2$, and $\pm 5/2$). In the presence of rhombic distortions the doublets are admixed and their energy separations are modified. An applied magnetic field (as in an EPR experiment) lifts fully the degeneracy of the three doublets. These effects can be described quantitatively by the following spin Hamiltonian, $\mathscr{H}$:

$$\mathscr{H} = D\left[S_z^2 - S(S+1)/3\right] + E\left[S_x^2 - S_y^2\right] + \beta \cdot \mathbf{g} \cdot \mathbf{H} \cdot \mathbf{S} \quad (1)$$

where $S = 5/2$ in the present case, and D and E are the axial and rhombic zero-field splitting parameters, respectively. The ratio E/D by an appropriate selection of axes is restricted to the range, 0 (axial symmetry) to 1/3 (rhombic symmetry).

Numerical solutions of Eq. (1) can be found, e.g., in Wickman et al. [13] and Sato and Kon [14]. The Zeeman term is generally small compared with the ligand field terms in Eq. (1) and is treated as a perturbation [14]. Up to second order corrections in the Zeeman term the effective g-values depend only on E/D. For most of the complexes examined here this is a good approximation and the E/D ratios corresponding to the observed g-values were obtained by polynomial interpolation [15] using the tabulated solutions in Ref. [14]. In a particular case, however, the D was sufficiently small that a higher order approximation was necessary.

The energy separations of the three doublets (in the order presented above) are respectively: $E_2 - E_1 = 2D + aD$ and $E_3 - E_1 = 6D + bD$, where a,b – up to second order corrections for the Zeeman term – are functions of E/D and vanish for $E/D = 0$. For $E/D = 0.12$, which is a typical value for some of the anions used in this study, $a = 0.23$ and $b = 0.15$, calculated by polynomial interpolation using the energy values tabulated by Sato and Kon [14]. As it has been discussed earlier [16,17], the resonances in the $g = 8$ region result from the g_y (or g_x depending on the sign of E) component of the $M_s = \pm 1/2$' doublet **g**-tensor, and the resonances in the $g = 5.5$ region result from the g_z component of the '$\pm 3/2$' doublet **g**-tensor. The temperature dependence of the intensity, I, of these components, recorded under nonsaturating conditions, follows the appropriate Boltzman distributions:

$$I_i \sim \frac{1}{T} \cdot \frac{\exp(-E_i/kT)}{1 + \exp(-E_2/kT) + \exp(-E_3/kT)} \quad (2)$$

where $i = 1$ for the $g = 8$ resonances and $i = 2$ for the $g = 5.5$ resonances. Since a,b are functions of E/D which is obtained from the positions of the peaks, an analysis of the temperature dependence of either the $g = 8$ or the $g = 5.5$ resonance with Eq. (2) can be used to determine the parameter D.

3. Results

Fig. 1 shows the anions used in the present study. Of these, bicarbonate is the physiological anion required for normal rates of electron flow. Formate has been used extensively as an inhibitor of bicarbonate binding [4,5,18,19]. The other anions can be classified either as monocarboxylate anions with a proximal polar group, keto (glyoxylate, pyruvate, Br-pyruvate, OH-pyruvate), hydroxy (glycolate, lactate), or as bicarboxylate anions (oxalate, malonate, malate) [8].

3.1. Effects on the $Q_A^- Fe^{2+}$ signal

The state $Q_A^- Fe^{2+}$ in PS II exhibits at least two alternative EPR signals; one at $g = 1.9$ and the other at $g = 1.84$ [20–22]. In a preliminary report we examined the effect of some of the anions on these signals produced by illumination at 200 K [9]. Extension of these studies to the other anions indicates that oxalate, Br-pyruvate, malate and malonate enhance the $g = 1.84$ form while glyoxylate, glycolate, lactate and pyruvate suppress it. Of the latter anions glyoxylate enchances the 1.9 resonance but the others do not give well defined resonances. OH-pyruvate appears to induce some damage to the reaction centre, as Mn is released and no signals from the iron-quinone complex can be detected.

3.2. Effects on the Fe^{3+} EPR signal

The non-heme iron of PS II can be oxidised to the Fe^{3+} form unlike its homologue in the purple bacterial reaction centres [16]. Oxidation of the iron results in the appearance of characteristic light-sensitive EPR signals at $g = 8.1$ and 5.6 [16]. We have examined the effect of the various anions on the Fe^{3+} EPR signals. Spectra of BBY samples treated with 5 mM $K_3Fe(CN)_6$ in the presence or absence (control) of the various anions are shown in Fig. 2 (pH 7.4) and Fig. 3 (pH 6.4). Variations both in the intensity and the position of the peaks are observed. Glycolate, glyoxylate lactate and pyruvate give strong signal intensities, while oxalate, formate (not shown) and to a smaller extent Br-pyruvate practically eliminate the Fe^{3+} signals. Malate appears to lower the signal intensity but no shift in the position of the peaks is observed. These effects are due to a decrease in the midpoint potential of the iron in the case of glycolate, glyoxylate, lactate and pyruvate and an increase in the case of oxalate, formate and Br-pyruvate, and are described below. The case of malate is less clear. It is likely that this anion raises the midpoint potential of the iron but binding is incomplete and the remaining signal intensity in Figs. 2 and 3 represents centres without bound malate. Malonate

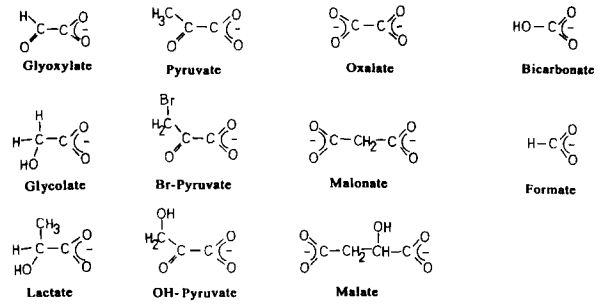

Fig. 1. The anions used in this study.

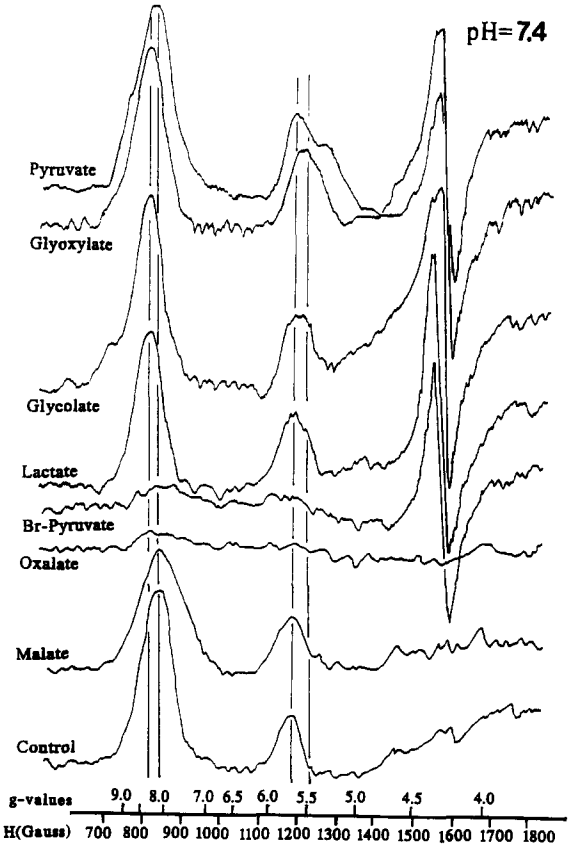

Fig. 2. Effect of the anions on the Fe^{3+} EPR signals. BBY samples (pH 7.4) were incubated with 5 mM $K_3Fe(CN)_6$ and no further additions (control) or with the indicated anions. The spectra are dark-illumination at 200 K difference traces. EPR conditions: $T = 4.1$ K, microwave power 31 mW and modulation amplitude 32 G.

(not shown) exhibits a similar behaviour. The anions also induce shifts in the resonances relative to the control and changes in the ratios of the areas of the $g = 8$ and $g = 5$ peaks. These effects reflect a change in the ligand field symmetry around the iron and are examined below.

A striking feature in some of the spectra of Fig. 2 is a light-sensitive split component at ca. $g = 4.3$. This resonance is broader than the commonly occurring $g = 4.3$ rhombic iron impurity component, and is not easily discernible in the spectra prior to subtraction of the light insensitive latter component. Plot on an expanded scale (not presented) shows that this new resonance has a resolved structure from which one can distinguish the g_x, g_y, and g_z components at 4.18, 4.24 and 4.34, respectively. The resonance bears strong similarities to the typical resonance of the Fe-transferrin-carbonate complex, (see Ref. [8] and references therein) as well as to the similar peak observed following replacement of the carbonate anion with monocarboxylate anions such as glycolate, pyruvate etc. [8]. We shall call this resonance 'split $g \sim 4.3$' in order to distinguish it from the impurity 4.3 component. The $g \sim 4.3$ peak is enhanced at higher pH values and becomes vanish-

ingly small below pH 7. As discussed below, this signal does not result from the same ligand symmetry which induces the signals in the $g = 5$ and 8 region. No such resonance could be detected in the absence of the anions in the pH range 5.5 to 9.0.

3.3. Effects on the zero-field-splitting parameters of the Fe^{3+}

A previous analysis of the Fe^{3+} spectra indicated that the g-values of 8.1 and 5.6 for the untreated sample correspond to $E/D = 0.11$ [16,17]. Also, analysis of the temperature dependence of the Fe^{3+} signals indicated that the zero field splitting parameter D was 2–3 K [16,17]. Examination of the spectra of Figs. 2 and 3 shows that glycolate, glyoxylate lactate and pyruvate induce small but distinct shifts in the g-values, which correspond to an increase in the value of the E/D parameter. Pyruvate with g-values of 8.7 and 5.3 has the strongest effect. This effect is not easily discernible in Fig. 2 due to incomplete binding of this anion at high pH. Careful examination of the spectra indicates also that the increase in E/D is accompanied by an increase in the ratio of the $g = 5$ relative to the $g = 8$ peak. This is predicted by the spin Hamiltonian analysis outlined in the previous section. With increasing E/D the transition probability within the middle Kramer's doublet increases relative to that of the first doublet. As a result, the relative intensity of the $g = 5$ resonance from the middle doublet is enhanced. An additional observation, particularly obvious in the case of pyruvate, is that the $g = 5.3$ peak is broader than in the control.

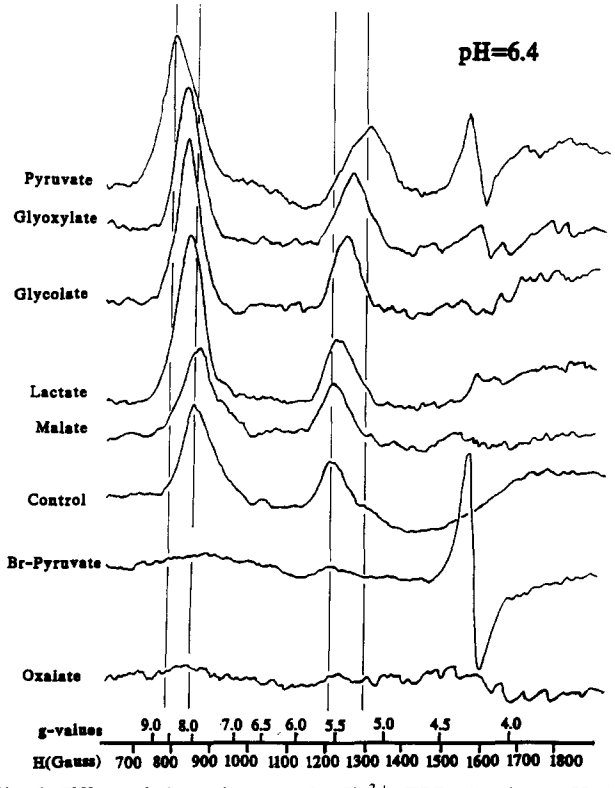

Fig. 3. Effect of the anions on the Fe^{3+} EPR signals at pH 6.4. Conditions as in Fig. 2.

Table 1
g-values [a] and corresponding spin-Hamiltonian parameters of the Fe^{3+} following ferricyanide treatment in the absence (untreated) or presence of the indicated anions

Additions	g_x	g_z^*	D (cm^{-1})	E/D
Untreated	8.07 [b]	5.60 [b]	1.4 ± 0.4 [c]	0.106
Lactate	8.18	5.57		0.110
Glycolate	8.21	5.52	1.0	0.118
Glyoxylate	8.32	5.48	0.8	0.124
Pyruvate	8.66	5.26	0.4	0.155

* Middle doublet.
[a] In addition to the indicated resonances all the anions in the table as well as Br-pyruvate exhibited at pH greater than 7 a split $g \approx 4.3$ resonance resulting from a minority of centres with nearly rhombic symmetry (see text).
[b] These values are more accurate than earlier estimates [16].
[c] Conservative estimate based on [16,17].

The g-values for some of the anions are given in Table 1. An analysis of the spectra in Figs. 2 and 3 with the spin Hamiltonian methods described above shows that these values correspond to E/D extending from 0.106 (control) to 0.155 (pyruvate). These values, also given in Table 1, indicate somewhat higher rhombicity when the anions are bound. The third digit accuracy in these estimates probably has a relative value, indicating the shifts induced by the various anions in the same type of preparation.

We have examined the effect of three of the anions on the D parameter. Fig. 4 shows the temperature dependence of the $g = 8$ peak, recorded under non-saturating conditions, for glycolate glyoxylate and pyruvate. The solid curves are the best fits to the experimental data with the indicated values of the parameters. The D-value for glycolate is 1.0 cm^{-1} which is within the range of the reported values for the untreated sample, 1.0 ± 0.3 cm^{-1} [17], 2–3 K (1.4–2.0 cm^{-1}) [16]. The D-value for glyoxylate is somewhat smaller, 0.8 cm^{-1} and that for pyruvate quite small, 0.4 cm^{-1}. The values of the D parameter are also included in Table 1.

As for the $g = 4.3$ resonance, which is part of most of the spectra at high pH, a simple consideration of the solutions of the spin Hamiltonian in Eq. (1) indicates that it cannot result from the same E/D and D parameters which induce the signals in the $g = 5$–8.6 region. This peak results from the middle Kramer's doublet for $E/D \sim 0.3$. A preliminary analysis indicates that all features of this resonance can be satisfactorily simulated with $E/D = 0.315$ and $D = 0.5$ cm^{-1}. The large E/D value as well as the different pH dependence shows that this resonance results from

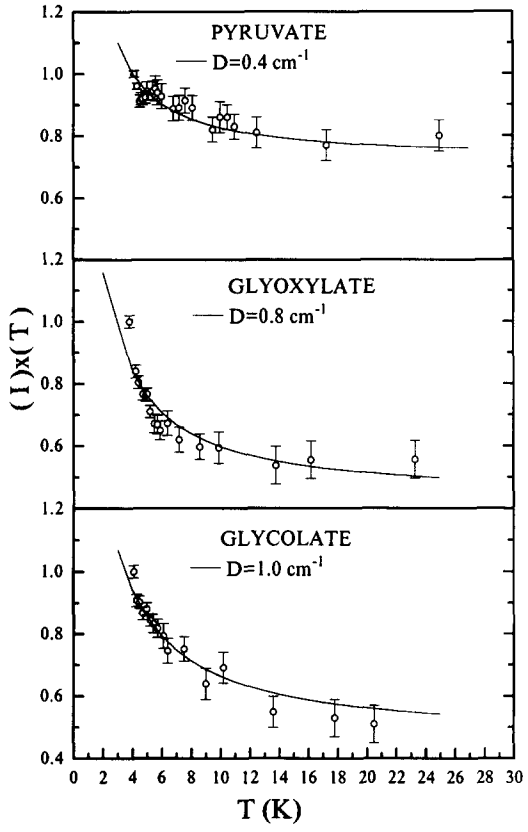

Fig. 4. Temperature dependence of the Fe^{3+} EPR signal intensity in the $g = 8.1$ to 8.7 region for three anions. EPR conditions: modulation amplitude 32 G, microwave power variable but below saturation. Continuous curves are theoretical simulations (see Materials and methods) with the indicated values of the D parameter and the values of E/D given in Table 1.

centres with different ligand geometry (nearly rhombic symmetry) at the iron site. The $g \sim 4.3$ peak is nearly isotropic and although it appears to be relatively intense compared to the resonances in the $g = 5$–8.6 region, it probably represents a small number of centres. An estimate based on the different transition probabilities and thermal occupations of the $g \sim 4.3$ and $g \sim 8$ resonances and the fact that the $g \sim 4.3$ peak is composite (the g_x component represents roughly 1/3 of the total intensity) indicates that, when comparing at 4.2 K, the $g_x \sim 8$ resonance with the overall intensity (peak to trough) of the $g \sim 4.3$ resonance, the latter should be divided by an approximate factor of 10. Examination of Fig. 2 indicates that, in all cases examined, the $g \sim 4.3$ does not represent more than $\sim 15\%$ of the total iron. This estimate is supported by the observation that appearance of this signal on increasing pH is accompanied by only a small decrease of the resonances in the $g = 5$–8.6 region.

3.4. Redox titrations of the Fe^{3+} EPR signals

The midpoint potential of the untreated Fe^{3+}/Fe^{2+} couple is pH dependent. At pH 7 the midpoint potential is 400 mV in thylakoids and varies with −60 mV/pH unit in the pH range ~ 5.3–8.5 [16,23,24]. In the BBY preparations used in the present study the midpoint potential was found to be somewhat higher: 420 mV at pH 7, while the pH dependence was preserved. The 20 mV difference is probably within experimental uncertainty but it may also be a consequence of the detergent treatment during the isolation procedure. This difference is not considered important for the conclusions of the present study. One of the important results, namely the lower midpoint potential of the iron in the presence of glycolate, was confirmed qualitatively with thylakoids (see below).

The spectra of Fig. 2 show that some of the anions allow oxidation of the iron and induce midpoint potentials which are even lower than the control sample. This is more apparent in Fig. 3. At pH 6.4 and an ambient potential of about 460 mV, close to the midpoint potential of the iron in the untreated system at this pH, some of the anions have fully developed signal intensities. The control sample, however, shows, as expected, about half the maximum signal intensity. In order to obtain more accurate estimates of the midpoint potential shifts and, also, examine possible pH dependencies, we have carried out redox titrations of the Fe^{3+} EPR signals in the presence of four of the anions in the pH range 6 to 7.5. The titration curves are shown in Fig. 5 and fitted with Nernst curves. Glycolate and lactate show a substantially lowered midpoint potential of about 340 mV with a weak pH dependence in the range 6 to 7.5 mV. Glyoxylate and pyruvate induce a midpoint potential of 390 mV at pH 6.1 and 330 mV at pH 7.2. The pyruvate spectra at pH 7.3 (see also Fig. 2) contained significant contributions from the spectrum of the untreated sample, indicating incomplete displacement of bicarbonate at this pH. Consequently, no accurate high pH titration data for this anion could be obtained. Careful deconvolution of the spectra at various E_h did, however, show that at this pH, too, the midpoint potential is significantly lower than in the control sample.

It was discussed in an earlier section that in the presence of oxalate, formate and perhaps malate, malonate and to a certain extent Br-pyruvate, no Fe^{3+} EPR signals are detected. This probably implies that in the presence of these anions the midpoint potential of the iron is significantly higher than the ambient potential, $E_h \sim 460$ mV, in these experiments. Strictly speaking, however, the absence of EPR resonances does not prove that the Fe^{3+} state is not formed. Although we have carefully screened the spectra, it is possible that the absence of these resonances may be due to severe broadening. We have accordingly examined the amount of electron acceptors beyond Q_A in samples oxidised with ferricyanide in the presence of oxalate or formate by studying the fluorescence relaxation. The fluores-

cence yield is high in the state Q_A^-, and in the presence of DCMU, it decays in the seconds time range, due to charge recombination with the donor side. In the presence of the Fe^{3+}, the fluorescence yield decays much faster, in 7–25 μs [16] due to electron transfer from Q_A^- to Fe^{3+}.

We examined the fluorescence yield at 500 μs after each of a series of actinic flashes, in chloroplast thylakoids poised at a potential approximately equal to the midpoint potential of the iron. Chloroplasts were incubated in the dark at 6.5 μg Chl/ml in a buffer containing 50 mM MES (pH 6.22), 0.3 M sorbitol, 10 mM NaCl and 5 mM $MgCl_2$ in the presence of either 30 mM bicarbonate, 100 mM oxalate or 100 mM formate. The samples were incubated for 0.5 h under

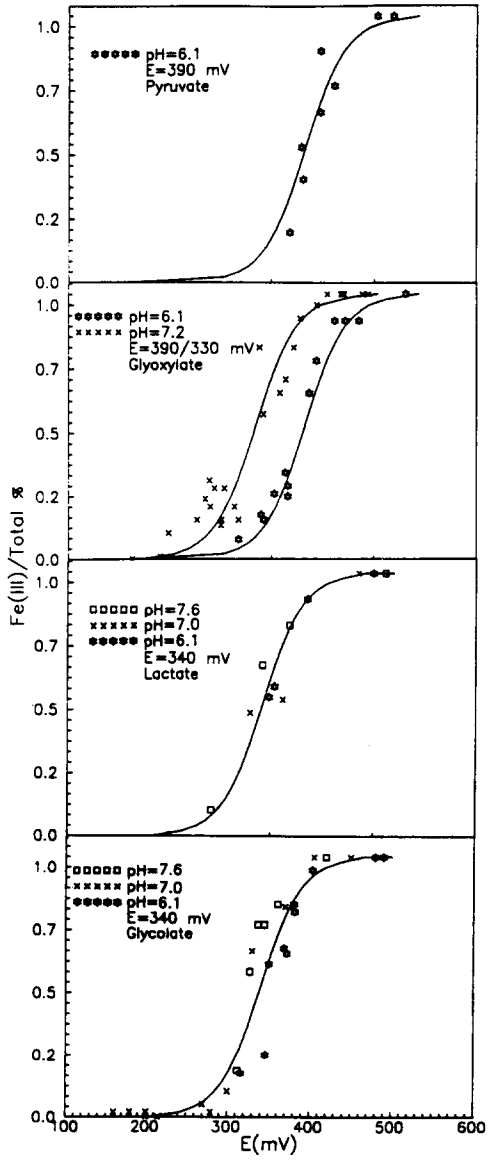

Fig. 5. Redox titrations of the Fe^{3+} EPR signals in the presence of the indicated anions at 40 mM concentrations each. Continuous lines are Nernst curves calculated for the indicated midpoint potentials.

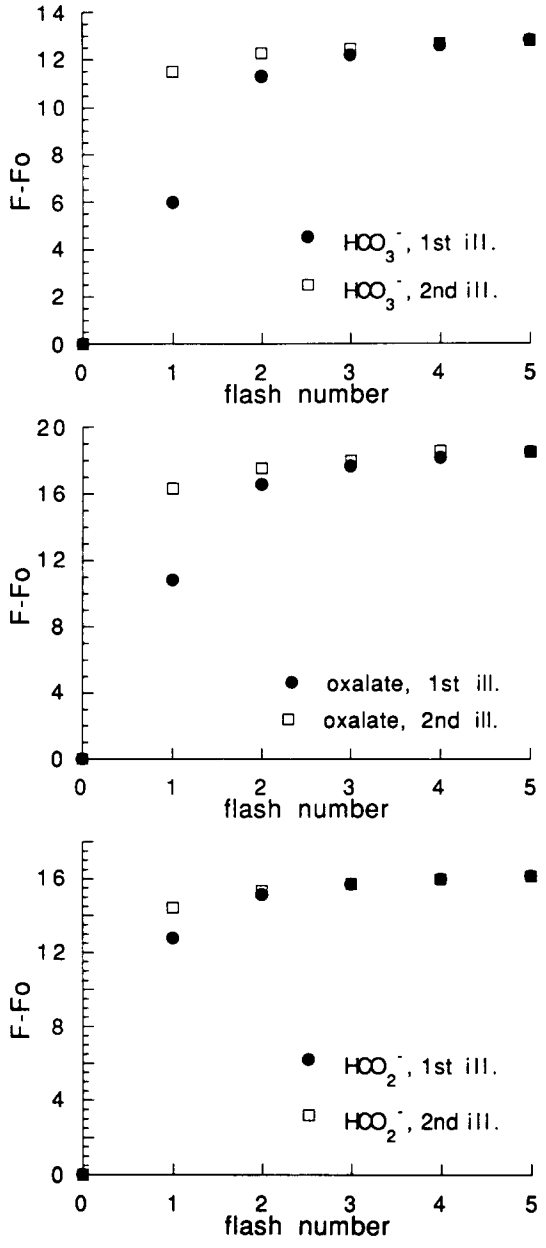

Fig. 6. Fluorescence yields at 500 μs after each of five flashes spaced 600 ms apart. A second series of flashes were given 1 min 15 s later. Chloroplasts, 6.5 μg Chl/ml, pH 6.22, were incubated with 100 μM ferricyanide in the presence of either 30 mM bicarbonate, or 100 mM oxalate, or 100 mM formate. DCMU, 40 μM, was added shortly before the flash series. The difference on the first flash is a measure of the amount of oxidised iron. See text for details.

these conditions, then 100 μM ferricyanide was added and incubation proceeded for 2 h. The samples were made 40 μM in DCMU and then given a series of five flashes at 600 ms between flashes. A second identical flash series was then given 1.25 min later. The fluorescence yield of the fifth flashes of each of the two series were normalised to each other (Fig. 6). Examination of Fig. 6 shows that the fluorescence yield increase between the first and second flash series is largest on the first flash with bicarbonate, less with oxalate and least

with formate. The oxidation of the iron by ferricyanide is slow [24] and therefore no appreciable oxidation occurs during the series of the five flashes or between the two flash series. Thus, the lower fluorescence yield following the early flashes in the first series reflects the rapid oxidation of Q_A^- ($\ll 500$ μs) by Fe^{3+} in the first series. The small increase in fluorescence yield during the second flash series reflects the flash yield of formation of Q_A^- with the non-heme iron in the Fe^{2+} state. The data of Fig. 6 show that approximately 50% of the centres have the iron oxidised in the presence of bicarbonate, while in the presence of oxalate or formate this percentage is 30% and 10% respectively. This implies that in the presence of formate or oxalate the redox potential of the iron is raised above its midpoint potential in the presence of bicarbonate.

Prompted by the fluorescence results, we have searched for Fe^{3+} EPR signals in the presence of formate and oxalate at relatively high redox potentials. BBY membranes at 100 μg Chl/ml were incubated for 4 h in the dark in a buffer containing 50 mM Mes (pH 6.0), 0.3 M sorbitol, 10 mM NaCl and 5 mM $MgCl_2$ in the absence (control sample) or presence of 200 mM formate or 100 mM oxalate. The redox potential of the solution was adjusted to 540 mV with ferricyanide. After incubation the sample was centrifuged and the pellet was resuspended in a buffer similar to the above, except with 1 mM ferricyanide, with subsequent incubation for 10 min. The final potential of the samples was approximately 510 mV.

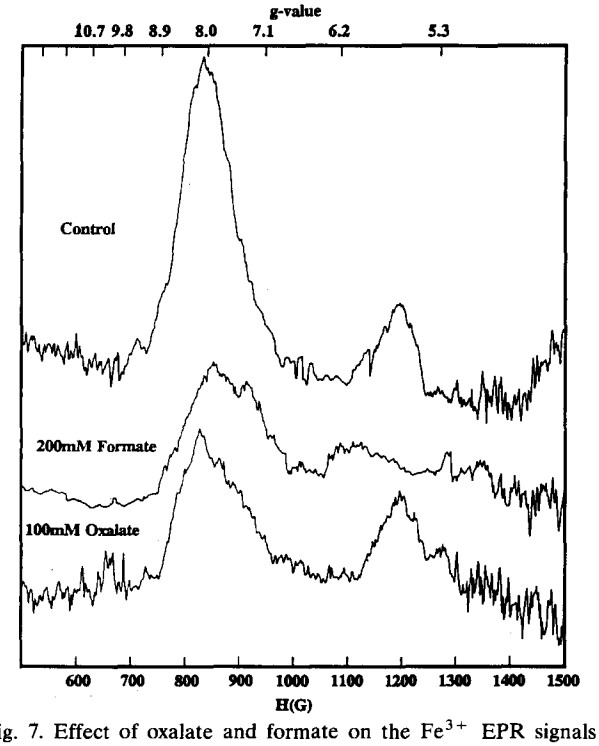

Fig. 7. Effect of oxalate and formate on the Fe^{3+} EPR signals in BBY samples (pH 6.0) poised with ferricyanide at $E_h \sim 510$ mV. Measuring conditions as in Fig. 2.

The EPR spectra are shown in Fig. 7 as dark-light difference traces. Compared with the control sample, which, as expected, has maximum signal intensity, the formate-treated sample is characterised by broader resonances with average g-values shifted to more axial positions $g = 7.8$ and 5.8. The integrated area of the spectrum is approximately 1/3 of the control. The oxalate-treated sample shows no appreciable shift in the position of the resonances although the spectrum appears somewhat broader. The area is approximately 50–60% of that of the control sample.

The above EPR results are in qualitative agreement with the fluorescence results and suggest that oxalate induces a modest increase, 20–40 mV, to the redox potential of the iron while formate raises the midpoint potential by more than 50 mV. These estimates should be considered as lower limits, since it cannot be excluded, particularly in the case of oxalate where the Fe^{3+} spectra resemble those of the control, that the spectra contain contributions from centres with the anions not bound.

The finding of an elevated midpoint potential of the iron in the presence of formate is in agreement with earlier literature. Radmer and Ollinger [26] have observed the absence of double hits in O_2 evolution upon flash excitation in the presence of ferricyanide and formate. Furthermore, Zimmermann and Rutherford [25] and Diner and Petrouleas [16] have observed no reductant-induced oxidation of the iron in the presence of formate. These and the earlier results, however, are in contradiction with a recent fluorescence study by Stemler and Jursinic [27]. These authors claim that formate does not modify the redox potential of the iron and that the effects they observe at high potential in the presence of formate, are due to a new donor-side component, D480. It seems that the present data and those of Stemler and Jursinic would be reconciled if we assume that D480 is the non-heme iron with formate bound.

The midpoint potentials in the presence of the various anions are given in Table 2.

3.5. Concentration dependence of the effects on the Fe^{3+} signals

The effects of the concentration of glycolate on the Fe^{3+} signals in BBY preparations were examined at 0.5, 1, 2, 5, 10, 20 mM (not shown). The dissociation constant (apparent, in competition with bicarbonate) was found to be $K_d \sim 0.5$ mM.

3.6. Light-induced oxidation of the iron in thylakoid membranes in the presence of glycolate

The non-heme iron can be oxidised by a number of exogenous quinones when reduced to their semiquinone

form by a single flash at room temperature [25] or a cycle of 200 K illumination followed by 30 s dark adaptation at 0°C [28]. The semiquinones Q_e^- transiently formed at the Q_B site are sufficiently strong oxidants to oxidise the non-heme iron. Studies of the pH dependence of this effect as well as examination of a number of quinones has indicated that the oxidising couple is Q_e^-/Q_eH_2 [25,28]. The native quinone, plastosemiquinone-9, fails to oxidise the iron even at pH 5.9 and it has been concluded that the effective potential for this redox couple is significantly lower in the reaction centre than in solution [28]. In the presence of glycolate the midpoint potential of the iron becomes sufficiently low such that the native quinone can possibly oxidise the iron by the mechanism described above. Indeed, in thylakoid membranes treated with 50 mM glycolate at pH 5.9, illumination at 200 K followed by 30 s dark adaptation at 0°C induces a Fe^{3+} signal. This signal is shown as the difference from the spectrum recorded immediately after the illumination at 200 K (Fig. 8b). The signal has a g-value of 8.21 consistent with bound glycolate and a less well defined resonance at $g \sim 5$–6. A similar signal but of smaller size (comparisons in this section are made in the $g \sim 8$ region since the broad $g = 5$–6 contributions are affected by background signals in these types of preparations) was present even prior to the illumination-dark adaptation protocol. This is also shown in Fig. 8a as a difference spectrum and is due to spontaneous oxidation of the iron. The size of this signal is clearly smaller and this has been confirmed with different samples. The oxidant in this case is not obvious but it could be Q_B^- which is known to be stably present in a fraction of centres in thylakoid preparations. Oxygen is also a likely alternative oxidant and this could explain the fact that spontaneously oxidised iron is present in BBY preparations treated with any of the anions which lower the midpoint potential of the iron. Oxidised cytochrome b_{559} could also be the terminal electron acceptor of the iron oxidation [29]. Fig. 8c shows for comparison the maximum Fe^{3+} signal produced in the same sample by treatment with 10 mM ferricyanide. This signal is approximately the sum of signals (a) and (b). This is expected if spontaneous oxidation is a slow process or if only a fraction of the centres contains Q_B^-. Accordingly, illumination at 200 K reduces the iron in the fraction of centres which had initially spontaneously oxidised iron and produces the state $Q_A^- Fe^{2+}Q_B$ in the larger fraction of centres which initially had the iron reduced. Subsequent dark adaptation for 30 s at 0°C is too short to allow spontaneous oxidation of the iron in the former fraction of centres but it allows secondary electron transfer and subsequent oxidation of the iron by Q_B^- [28] in the latter fraction of centres.

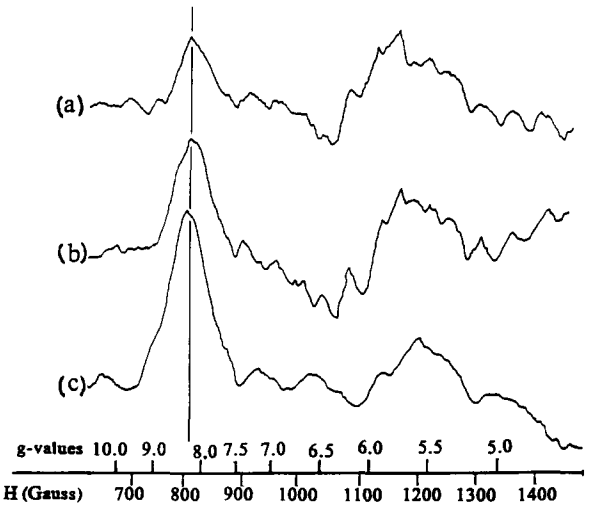

Fig. 8. (b) Light-induced oxidation of the iron in thylakoid preparations (pH 5.9, 3 mg Chl/ml) in the presence of 50 mM glycolate induced by 200 K illumination followed by 30 s dark adaptation at 0°C; the native plastosemiquinone here is the oxidant. (a) spontaneously oxidised iron in the same sample existing prior to the illumination. (c) Maximum Fe^{3+} signal in a similar sample treated with 10 mM ferricyanide. Dark −200 K illumination difference EPR spectra were recorded as in Fig. 2.

Table 2
E_m(NHE) values for the Fe^{3+}/Fe^{2+} couple at two pH's and pK_a values of the various anions. The listed midpoint potentials were obtained by titrating the major Fe^{3+} EPR resonances in the g-regions 8–9 and 5–6. The high pH resonance at $g = 4.3$, resulting from a minority of centres, titrated with somewhat different E_m values, but no accurate data were obtained.

Addition	E_m (NHE)		
	pH 6.1	pH 7.5	pK_a
Glycolate [a]	340	340	3.83
Lactate [a]	340	340	3.86
Glyoxylate	390	330	3.32
Pyruvate	390	330 [b]	2.49
OH-Pyruvate	> 500	> 500	–
Br-Pyruvate	> 500	> 500	–
Oxalate	> 460	> 460	1.27, 4.29
Malate [b]	> 500 [b]	> 500 [b]	3.40, 5.13
Formate	> 500	–	3.75

[a] In the presence of glycolate and lactate a weak pH dependence may have been obscured by experimental uncertainties.
[b] These E_m estimates are tentative due to incomplete binding of the anions.

4. Discussion

The present results demonstrate that a number of carboxylate anions affect the EPR signals associated with the iron-quinone complex, the ligand symmetry at the iron site as reflected in the Fe^{3+} EPR spectra, and the redox properties of the iron. The implications and the possibility that these anions bind as iron ligands are discussed below.

4.1. $Q_A^- Fe^{2+}$ EPR spectra

The effects of the various anions on the $Q_A^- Fe^{2+}$ signals are rather small (see also Ref. [9]). Oxalate and to a smaller extent Br-pyruvate and malate enhance the $g = 1.84$ signal albeit with somewhat different characteristics than the control. Glyoxylate, glycolate, pyruvate and lactate diminish the $g = 1.84$ signal and appear to favour the $g = 1.9$ resonance. The $Q_A^- Fe^{2+}$ signal in PS II is, however, intrinsically small and the observed changes are difficult to quantitate. The most prominent effect is that of oxalate, which in some preparations induces a large enhancement of the $g = 1.84$ signal. This is reminiscent of the large increase in the $g = 1.84$ signal intensity induced by formate binding [30], but the effect of oxalate appears to be smaller.

4.2. Effects on the Fe^{3+} spectra

The present anions have distinct effects on the Fe^{3+} EPR resonances. Compared to the control with g-values of 8.07 and 5.60, glycolate shifts the resonances to 8.21 and 5.52 and pyruvate to 8.66 and 5.26. These shifts indicate somewhat greater rhombicity (higher E/D values) of the iron environment in the presence of these anions. In addition to the above shifts, a striking feature of the Fe^{3+} spectra in the presence of most anions is a nearly rhombic resonance at $g \sim 4.3$ at alkaline pH. This resonance shows strong similarities to the characteristic EPR spectrum of transferrin (Ref. [8] and references therein). It is unlikely that this signal results from any species other than the acceptor side non-heme iron. Heme iron (cyt b_{559}) could not give such a resonance. In addition, the $g = 4.3$ signal, like the other non-heme Fe^{3+} resonances, disappears upon low temperature (200 K) illumination implying that this iron species acts as an electron acceptor.

An interesting observation is that the $g = 4.3$ signal decreases drastically below pH 7 except for Br-pyruvate. This could reflect the acidity of the C-2 and C-3 protons of the carboxylate anions. In a discussion of electrophilic catalysis in the enzyme catalysed abstraction of protons from carbon acids, Gerlt et al. [31] propose that protonation or metal coordination of the enolate form of carboxylic acids decreases the pK_a of the a-carbons to approach those of the active site bases, where the difference in pK_a is 2–5. Thus in enzymes like glycolate oxidase and ferricytochrome b_2 where the substrates are glycolate and lactate, respectively, the pK_a of the active site bases are thought to be about 7 and 5.3, respectively. Given the turnover times of these enzymes the pK_a of the C-2 proton would be about 5 units higher than that of the active site base or 10–12. If in addition the proximal groups are ligated and there are cationic charges nearby (D2-Lys264 and D2-Arg265), then the pK_a of the C-2 and C-3 protons could be lowered still further. Finally Br-pyruvate, with the electron withdrawing halogen, would be expected to show a pK_a of the C-3 proton lower still than pyruvate, consistent with the observations of Figs. 2 and 3. Some structural implications of the present results are discussed in the companion paper [32].

4.3. The ligand field parameters of the iron

There is a progressive decrease of the D parameter and a relatively smaller increase in the E/D value in the series, untreated, lactate, glycolate, glyoxylate, pyruvate (Table 1). This implies that the decrease of D is accompanied by a smaller decrease of E. Therefore, although the EPR spectra appear progressively more rhombic (due to the increasing value of E/D) the overall deviation from octahedral symmetry is probably smaller in the case of pyruvate than in the untreated sample. The same probably holds true for the $g \sim 4.3$ resonance, but a better understanding of this resonance has to await a more detailed characterisation including orientation studies.

It is interesting to note that molecules that bind at the Q_B site such as p-benzoquinones, dinoseb or o-phenanthroline induce a strong decrease in the E/D parameter while D-value is least affected [16]. This probably implies that the two classes of molecules induce distortions along different axes of the iron octahedron.

4.4. Effects on the midpoint potential of the iron

The effects of the present anions on the midpoint potential of the iron are quite interesting. We found earlier that molecules that bind to the Q_B site also affect the midpoint potential of the iron. However in all cases examined, including DCMU, o-phenanthroline, atrazine, phenyl-p-benzoquinone the midpoint potential became more positive [16,24,28]. In the present case some of the anions, glycolate, lactate, glyoxylate, and pyruvate reduce substantially the midpoint potential of the Fe^{3+}/Fe^{2+} couple (see Table 2). On the other hand, formate and oxalate, and probably Br-pyruvate, malate and malonate raise significantly the midpoint potential.

The midpoint potential of the iron in the presence of glycolate is so low that light-induced oxidation of the iron is possible in thylakoids with the native plastosemiquinone couple Q_B^-/Q_BH_2 [28], acting as the oxidant. No iron oxidation is observed under similar conditions in an untreated sample. Since the midpoint potential of the untreated iron in thylakoids at pH 6 is about 460 mV while that in the case of glycolate is 340, this restricts the midpoint potential of the Q_B^-/Q_BH_2 couple at pH 6 to $\gg 340$ and $\ll 460$ mV. A conserva-

tive estimate would be 400 mV. Assuming a −120 mV/pH unit dependence, the operating midpoint potential of the Q_B^-/Q_BH_2 couple at pH 7 should be approximately 280 mV, well below the reported value of 388 mV in solution [28] and in good agreement with a previous indirect estimate [33].

Previous studies showed that some of the iron undergoes spontaneous oxidation in untreated thylakoids [24] and in *C. reinhardtii* membranes [16]. In untreated BBY preparations we have not observed spontaneous oxidation, presumably because of the somewhat higher midpoint potential of the iron in this type of preparation. In the presence of the anions which induce a low redox potential (glycolate, glyoxylate, etc.) spontaneous oxidation is also observed in BBY preparations. The oxidant is very likely molecular oxygen [24] or Q_B^- which may be stably present in a fraction of the centres. However, high potential cyt b_{559} [29] should not be excluded as an oxidant of the non-heme iron.

One correlation that appeared in the course of this work is that those anions (e.g. oxalate, formate, malate, bromopyruvate) that show a g-value of 1.84 for the $Q_A^-Fe^{2+}$ EPR signal are also those that raise the E_m of the Fe(III)/Fe(II) couple. A similar trend is also observed in the presence of bicarbonate alone, but as a function of pH. The midpoint potential of the untreated iron (Q_{400}) shows a pH dependence of approx. −60 mV/pH unit [16,23] in the pH range bound by the pK values $pK^O = 5.3$ and $pK^R = 8$ (O, R denote iron oxidised, reduced respectively) [24]. In addition in lowering the pH below 7, an increasing fraction of PS II centres is characterised by a high midpoint potential of the iron (much higher than the −60 mV/pH unit increase) [28] and a $g = 1.84$ signal in the state $Q_A^-Fe^{2+}$ [21]. It is interesting that no such heterogeneity is observed with the present anions and it likely reflects changes associated with bicarbonate itself. The pH dependence of the Fe(III)/Fe(II) couple (−60 mV/pH unit) implies a deprotonation event coupled to the oxidation of the iron. This dissociation could occur on the ligand itself or on a nearby amino acid residue or set of residues. Were the pK_as of the ionisable group(s) to be shifted either up (high pK_a state) as in the case of oxalate or down (low pK_a state) as in the case of glycolate, then the former would raise the E_m and the latter would lower it, respectively. If, in addition, the high pK_a state were to show a $Q_A^-Fe^{2+}$ g-value of 1.84 and the low pK_a state 1.90, then the correlation between the g-value and the E_m of the iron couple would hold for both the various carboxylate anions and the pH-dependent heterogeneity in the presence of bicarbonate alone. This model is further substantiated by the very weak pH dependence for the oxidation of the non-heme iron in the presence of glycolate or lactate, presumably because the pK_a of the ionisable site(s) appears now to be < 6.1 with the iron reduced. This model encompasses an earlier proposal by Nugent and co-workers [34] where the g-value of the $Q_A^-Fe^{2+}$ EPR signal was proposed to be determined by the presence of bound bicarbonate. We see here that other anions can substitute for bicarbonate in this role. The earlier model is a subset of the present one in that bound bicarbonate would determine the pK_a of the ionisable group(s) that in turn determines the g-value for $Q_A^-Fe^{2+}$ and the E_m of the iron couple. Whether the two forms of the $Q_A^-Fe^{2+}$ couple differ solely in the pK_a state of some ionisable group or by a difference in the coordination environment as well is still unclear.

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