Manganese-quinone interactions in the electron acceptor region of bacterial photosynthetic reaction centres

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A wild-type strain of *Rhodopseudomonas sphaeroides* was found to have manganese, instead of the expected iron, associated with the quinone acceptor complex in a high proportion of its photosynthetic reaction centres. Almost homogeneous reaction centre preparations containing either of these metals were obtained by appropriate depletion or enrichment of the metals in the growth media. An EPR signal attributed to reaction centre bound manganese was present in the dark in manganese-containing centres. This signal was absent under conditions where either Q_A or Q_B were in the semiquinone form. New EPR signals attributed to Q_A^- Mn and Q_B^- Mn are reported.

Photosynthesis *Manganese-semiquinone* *ESR* *Iron-semiquinone*

1. INTRODUCTION

The reduced form of the primary quinone acceptor, Q_A, in purple bacteria gives rise to a broad EPR signal at $g = 1.8$ [1,2]. The signal arises from a magnetic interaction between the semiquinone and a ferrous iron atom [3]. The same iron atom interacts with the semiquinone form of the secondary acceptor Q_B also giving rise to an EPR signal at $g = 1.8$ [4–8]. Despite recent advances [9,10], the nature of the interaction between the semiquinones and the iron is not fully understood and the role of the iron atom is still not clear. In fact it has been reported recently that the iron atom can be removed while electron transfer from Q_A to Q_B still proceeds, albeit with slower kinetics [11].

A potentially interesting approach, which may provide further information on the structural environment and the function of the iron, is to replace iron with other metals. The iron atom can be replaced by manganese when bacteria are grown on iron-depleted, manganese-enriched media. This seems to have little or no effect on the electron transfer reactions [12–14]. In the first report of manganese-containing reaction centres (manganese was present in 30% of the centres), a light-modulated EPR spectrum was reported [12]. This was thought to arise from Q_A^- interacting with manganese. In a more recent study the iron was replaced by manganese in 58% of the centres and although the presence in the dark of low-symmetry Mn^{2+} EPR signals was briefly described, no light-induced signals were observed [14].

By using a strain of *Rhodopseudomonas sphaeroides* in which manganese appears to replace partially iron even under normal growth conditions [15] we have obtained reaction centres in which iron is almost completely replaced by manganese. In these reaction centres we have found EPR signals arising from manganese in the presence and absence of interactions with Q_A^- and Q_B^-.

Abbreviations: BPh, bacteriopheophytin; DAD, diaminodurene; P_{865}, primary electron donor; Q, ubiquinone; LDAO, lauryldimethylamine *N*-oxide; PS, photosystem

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2. MATERIALS AND METHODS

R. sphaeroides wild-type (Y strain) cells were grown in semi-synthetic media containing various amounts of Fe and/or Mn. In all cases the growth medium contained per litre final volume: 20 mM K phosphate buffer (pH 6.8), 3.5 g ammonium succinate, 0.6 g Na acetate, 1 g Na glutamate, 1 mg nicotinic acid, 0.5 mg thiamine, 10 μg biotin, 2.5 mg nitrilotriacetic acid, 289 mg $MgSO_4 \cdot 7H_2O$, 66 mg $CaCl_2 \cdot 2H_2O$, 0.185 mg $NH_4Mo_7O_{24} \cdot 4H_2O$, 11 mg $ZnSO_4 \cdot 7H_2O$, 0.39 mg $CuSO_4 \cdot 5H_2O$, 0.25 mg $Co(NO_3) \cdot 6H_2O$, 0.18 mg $Na_2B_4O_7 \cdot 10H_2O$. To 1 l of this basal medium Fe and/or Mn salts were added as follows. For medium A ('normal'): 7 mg $FeSO_4 \cdot 7H_2O$, 1.54 mg $MnSO_4 \cdot H_2O$ (these are the amounts found in the Hutner modified medium of Cohen-Bazire et al. [16]). For medium B ('Mn-enriched'): 30.8 mg $MnSO_4 \cdot H_2O$, no Fe salt. For medium C ('Mn-depleted'): 7 mg $FeSO_4 \cdot 7H_2O$, no Mn salt.

The strain was maintained in medium A. Normal cultures were grown under ~8000 lux in this medium in 10-l bottles, at 30°C, with magnetic stirring and bubbling of N_2-5% CO_2, and harvested after 24 h. To enrich the cultures in Mn, they were subcultured 3 times in medium B in completely filled 500-ml bottles, at 30°C, under 5000 lux. The last stage of the enrichment series served as inoculum for a 10-l bottle, which was grown as above and harvested after ~4 days. To deplete the cultures of Mn, the same procedure was followed using medium C.

Reaction centres were isolated with LDAO and purified as in [17]; an additional DEAE-cellulose chromatography with a linear NaCl gradient was performed for preparations from normal cells. They were concentrated under N_2 on an Amicon XM 30 membrane in 10 mM Tris-HCl, 0.1 mM EDTA, 0.1% LDAO buffer, pH 8.0. The amounts of Fe and Mn bound to these preparations were measured by atomic absorption as in [15]. Kinetic measurements of absorbance changes after flash excitation were performed on a home-made single-beam spectrophotometer.

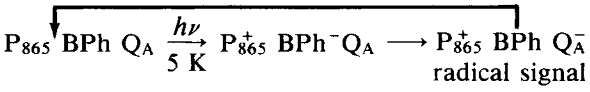
EPR spectra were obtained using a Bruker ER-200$_T$-X-band spectrophotometer fitted with an Oxford Instruments liquid helium cryostat and temperature control system; samples were illuminated in the cryostat using an 800 W projector. The same projector, but at an attenuated voltage, was used to excite samples at room temperature in a water bath and while freezing. Flashes at room temperature were provided by a Quantel YAG laser (80 mJ, 15 ns pulse, 530 nm). Samples were frozen approx. 1 s after flash excitation.

3. RESULTS

Reaction centres were prepared from *R. sphaeroides* grown on normal, manganese-depleted and manganese-enriched media. The relative proportions of iron and manganese present in each kind of centre were measured by atomic absorption spectroscopy. As previously reported reaction centres grown on normal medium had manganese present in 60% of the centres [15]. Growth on manganese-enriched medium resulted in an increase in the proportion of manganese-containing centres to 86% while growth on iron-enriched media resulted in centres containing almost 100% iron.

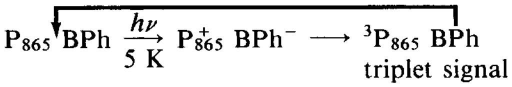
The EPR spectra obtained from these preparations showed large light-induced $g = 1.8$ signals in iron-containing centres (fig.1, inset b), smaller $g = 1.8$ signals in normal mixed iron-manganese centres (fig.1, inset a) and no detectable $g = 1.8$ signals in Mn-containing centres (fig.1, inset c). Thus, as expected, the amplitude of the semiquinone iron signal from Q_A^- at $g = 1.8$ reflects the amount of iron present in the centres.

It is possible that the Q_A^- Fe signal at $g = 1.8$ could be lost due to loss of Q_A during sample preparation. To test this possibility low-temperature photochemistry was monitored in all samples (see [18]). If Q_A is present in all samples, illumination at low temperature results in the following reaction:



$$P_{865}\,BPh\,Q_A \xrightarrow[5\,K]{h\nu} P_{865}^{+}\,BPh^{-}Q_A \longrightarrow \underset{\text{radical signal}}{P_{865}^{+}\,BPh\,Q_A^{-}}$$

If Q_A is absent or reduced, illumination at low temperature results in the following reaction:



$$P_{865}\,BPh \xrightarrow[5\,K]{h\nu} P_{865}^{+}\,BPh^{-} \longrightarrow \underset{\text{triplet signal}}{{}^{3}P_{865}\,BPh}$$

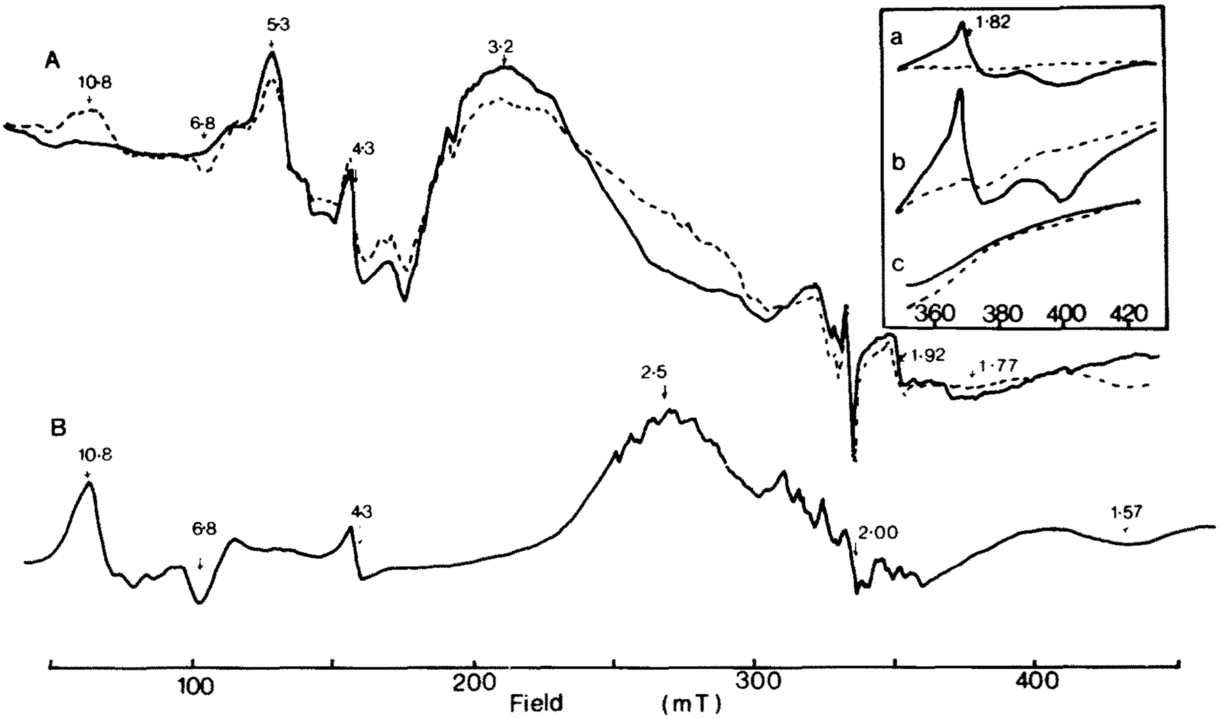


Fig.1. EPR spectra from manganese-containing reaction centres. (A) No additions, spectrum recorded in darkness (——), spectrum recorded under illumination (– – –). (B) Incubated under O_2 free argon in the presence of sodium dithionite (50 mM) in the dark at 20°C for 10 min. 0.1% Triton X-100 was added to the sample before dithionite addition to keep the reaction centres in solution after the LDAO precipitated with dithionite. The spectrum was recorded in the dark. EPR spectrometer settings were: microwave frequency 9.44 GHz, microwave power 8 dB down from 200 mW (≃35 mW), modulation amplitude 20 G, temperature 40 K. The gain for B was half of that used for A. The inset shows the photoinducible semiquinone iron signal in samples of variable manganese and iron content. (a) Normal, (b) Mn-depleted, (c) Mn-enriched. For the inset broken lines were recorded in the dark, solid lines were recorded under illumination. EPR conditions were as above except the temperature was 4.5 K. Reaction centre concentration was 130 μM for A and B and also for (c) in the inset and was 30 μM for (a) and (b) in the inset.

It was found that large photoinduced P^{+}_{865} radical signals (at $g = 2.002$) of comparable amplitudes were present in all samples while no triplet could be detected. This demonstrates that no Q_A was lost in sample preparation.

Another possible explanation for loss of the $g = 1.8$ signal could be that iron is lost but not functionally replaced by manganese. Under such circumstances a simple light-induced radical at $g = 2.0045$ from uninteracted Q_A^- might be expected. No such signal could be observed. It thus seemed likely that the iron is replaced by the manganese and that the semiquinone is magnetically coupled to the manganese.

A search was carried out for signals arising from manganese in the reaction centre. Fig.2A (solid line) shows an EPR spectrum recorded in the dark in manganese-containing reaction centres. A number of resonances were present in these samples which were absent in samples that contained iron. These features are at approx. $g = 5.3$, $g = 3.9$, $g = 3.2$ and $g = 1.77$.

As discussed above illumination of reaction centres at liquid helium temperature should result in formation of P^{+}_{865} and Q_A^-Mn. Under illumination P^{+}_{865} was indeed formed (fig.2, inset a) and there were differences induced at a number of field positions in the EPR spectrum (fig.1A, dotted line). In particular a new peak at $g = 10.8$ was formed, absorption decreases at $g = 6.8$, $g = 5.3$ and $g = 3.2$ occurred and an increase in absorption was evident at $g = 2.5$. The amplitude of these photoinduced

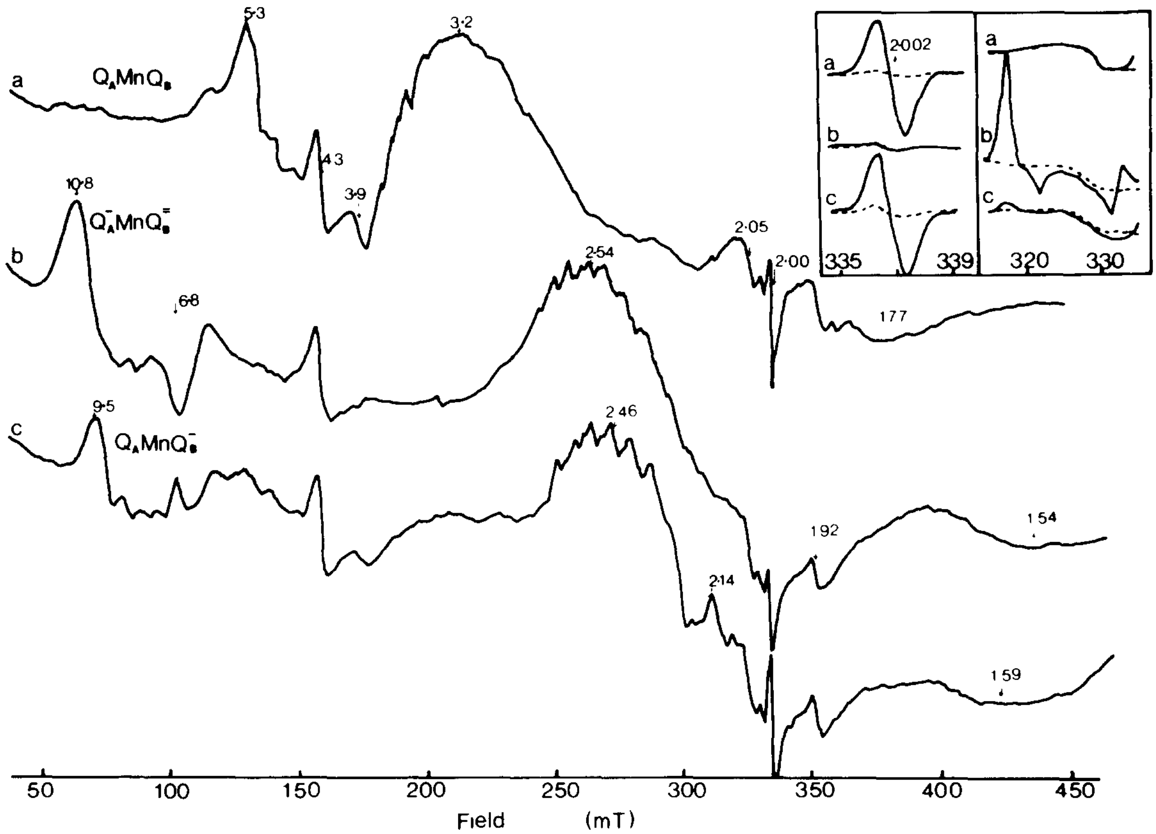


Fig.2. EPR spectra of manganese in the presence and absence of Q_A^- and Q_B. All samples contained DAD (10 mM) and ascorbate (50 mM). The EPR spectra were recorded in darkness using the settings described in fig.1. (a) Dark-adapted, (b) illuminated at 20°C and frozen under illumination, (c) excited by a single flash at room temperature. The inset shows photoinduced signals at low temperature. The left panel shows P_{865}^+ (——, under illumination; – – –, in the dark); instrument settings, microwave power 60 dB down from 200 mW, modulation amplitude 2 G, temperature 40 K. The right panel shows $^3P_{865}$ (——, under illumination, – – –, dark); instrument settings, microwave power 20 dB down from 200 mW, modulation amplitude 2 G, temperature 40 K.

changes was dependent upon the light intensity and it was found that the maximum intensity available was not saturating.

To verify that the changes observed at low temperature were due to photoreduction of Q_A^-, two techniques were used to reduce Q_A prior to freezing. Firstly, samples were chemically reduced by sodium dithionite. The presence of Q_A^- was verified by the observation of a large P_{865} triplet and no P_{865}^+ radical signals. Fig.1B shows the EPR spectrum obtained from a chemically reduced sample. All of the features of the signal present in the dark (fig.1A) were absent. Instead a new signal with features at $g = 10.8$, $g = 6.8$, $g = 2.5$ and $g = 1.57$ was present. These features correspond closely with those of the signal photoinduced at low temperature (fig.1A, dotted line). This signal is attributed to Q_A^-Mn.

It is of note that the features at $g = 4.3$ (rhombic ferric iron), $g = 2.05$ (Cu^{2+}) are contaminants present in the baseline and the $g = 1.92$ signal (perhaps an iron-sulphur centre) is probably an impurity in the preparation since it is present in most samples but in varying amounts and it shows no photochemistry. The complex spectrum around $g = 2$ in fig.1B is attributed to a small amount of hexacoordinated Mn^{2+}. This signal is not present in the spectrum of photoreduced Q_A^-Mn (see fig.2b) and is considered to reflect centres damaged by the dithionite treatment.

The second method used to reduce Q_A was to freeze samples under illumination in the presence of electron donors (DAD, ascorbate) but in the absence of added acceptors. Fig.2b shows the spectrum obtained when a sample was treated in this way. The spectrum has all the features observed for Q_A^-Mn when observed by reversible photoreduction at low temperature (fig.1A, dotted line) or by chemical reduction (fig.1B). The redox state of the reaction centre was verified by monitoring P_{865}^+ and $^3P_{865}$ signals and it was found that Q_A was completely reduced (fig.2, inset b).

Both of these methods for reduction of Q_A^-Mn also reduce Q_B when it is present. However, Q_B readily undergoes a second reduction step and so becomes EPR silent. Thus, in figs 1B and 2b no contribution from Q_B^-Mn is expected. However, a single flash given in the presence of exogenous donors should result in Q_B^- formation. Fig.2c shows a spectrum obtained after a single flash at room temperature. The signal present in dark-adapted samples was completely absent. Instead a new signal is present which resembles the Q_A^-Mn signal. In several respects this signal differs from that of Q_A^-Mn: (i) the lowest field feature is at $g = 9.5$ rather than at $g = 10.8$; (ii) the $g = 6.8$ feature is observed as a spike rather than a trough; (iii) the broad feature around $g = 2.5$ seems to be shifted to higher field and the hyperfine splitting on this peak is better resolved; (iv) a new feature is present at $g = 2.14$; (v) the broad trough at around $g = 1.55$ seems to be shifted to higher field.

Monitoring of the P_{865}^+ and $^3P_{865}$ signals showed that Q_A was oxidized after the flash excitation (fig.2, inset c). This signal is attributed to Q_B^-Mn.

To verify this assignment experiments were carried out giving a series of flashes at room temperature to samples in the presence of exogenous donors (DAD, ascorbate) and acceptors (ubiquinone 6). Under these conditions it has been shown previously that Q_B^- forms on odd-numbered flashes and is double reduced on even-numbered flashes, working as the two-electron gate of electron transfer out of the reaction centre [4,19]. Fig.3 shows that the signal present in the dark which disappears on the 1st flash, reappears on the 2nd flash and disappears again on the 3rd flash. The Q_B^-Mn signal is formed on the 1st flash, disappears on the 2nd and reappears on the 3rd. Monitoring of P_{865}^+ showed that Q_A remained oxidized throughout the flash series. The characteristic oscillation behaviour shown in fig.3 is clear evidence that the signal induced after the 1st (and 3rd) flash is due to Q_B^-Mn.

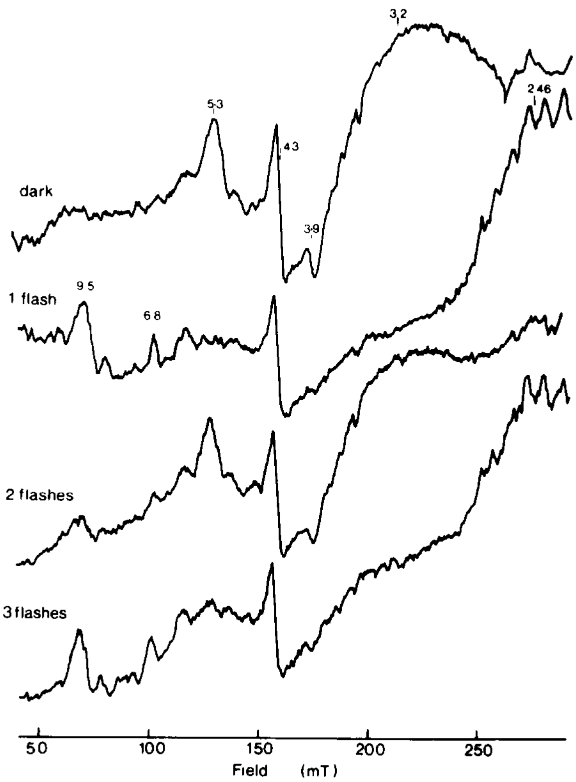


Fig.3. Oscillations of Q_B^-Mn with flash number. All samples contained DAD (10 mM) ascorbate (50 mM) and ubiquinone 6 (approx. 0.5 mM). Flashes were given at room temperature and samples were frozen within approx. 1 s of flash excitation. Reaction centre concentration was 40 μM in all samples. EPR conditions were as in fig.1.

Fig.4 shows a comparison of the broad peak at close to $g = 2.5$ in Q_A^-Mn and Q_B^-Mn. Clearly defined hyperfine splitting is present in both spectra but is better resolved for Q_B^-Mn. The hyperfine peaks exhibit a marked structure making it difficult to measure accurately the peak-to-peak splitting, however, the average splitting was approx. 71 and 73 G for Q_A^-Mn and Q_B^-Mn, respectively. In both cases there were 6 definite peaks present. This hyperfine splitting is characteristic of coupling with the Mn^{55}, spin 5/2 nucleus and is compatible with that expected from Mn^{2+}.

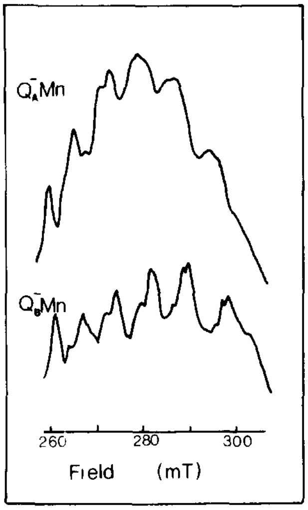


Fig.4. Hyperfine splitting on the Q_A^-Mn and Q_B^-Mn signals. The Q_A^-Mn signal was obtained from a sample reduced by sodium dithionite as in fig.1B, while Q_B^-Mn was generated by flash illumination as in fig.2B.

Signals identical to those shown in fig.2 were observed under the same conditions in reaction centres from normally grown cells except that the signal amplitudes were decreased corresponding to the lower manganese (and higher iron) content (not shown). None of these signals were detected in manganese-depleted centres.

4. DISCUSSION

The results reported above show that manganese can replace iron in purified bacterial reaction centre of *R. sphaeroides* in agreement with previous work [12–14]. However, this work shows that, at least in this strain of bacteria, a significant proportion of centres containing Mn would appear to be the normal situation.

A number of EPR signals have been discovered in centres where manganese is present. In the dark the manganese gives rise to a complex signal that is presumably the same as that briefly described earlier [14]. The fact that this dark Mn signal is totally lost when either Q_A or Q_B is in the semiquinone form is clear evidence that this signal arises from the specific bound manganese within the reaction centre. The new signals attributed to Q_A^-Mn and Q_B^-Mn are similar in overall form yet slight differences show that the coupling of the Mn with each of the quinones is not identical. This is also the case with Q_A^-Fe and Q_B^-Fe in *R. sphaeroides* [5,6,8], *R. viridis* [7] and also probably in PS II [20].

The only previously reported spectrum of semiquinone manganese signals was obtained using light modulation [12], however it seems likely that this spectrum was due to both the loss of the dark manganese signal and the formation of Q_A^-Mn. The new semiquinone manganese signals reported here should now be the subject of detailed EPR analysis to obtain information on the electronic structure of the manganese in the reaction centre. This might also lead to a better understanding of the function of the metal in this region of electron transport.

The close analogy between the acceptor side of purple bacteria and PS II (review [21]) leads to the interesting speculation that in PS II iron might be naturally substituted by manganese under some conditions or in some species. This would have an influence on the amplitude of the Q_A^-Fe signal observable in PS II and also would be important in trying to understand stoichiometries of manganese in the O_2-evolving enzyme previously thought to be the only functional site for manganese in PS II.

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REFERENCES

[1] Feher, G., Okamura, M.Y. and McElroy, J.D. (1972) Biochim. Biophys. Acta 267, 222–226.
[2] Leigh, J.S. and Dutton, P.L. (1972) Biochem. Biophys. Res. Commun. 46, 414–421.
[3] Okamura, M.Y., Isaacson, R.A. and Feher, G. (1975) Proc. Natl. Acad. Sci. USA 72, 3491–3495.
[4] Wraight, C.A. (1977) Biochim. Biophys. Acta 459, 525–531.
[5] Wraight, C.A. (1978) FEBS Lett. 93, 283–288.
[6] Okamura, M.Y., Isaacson, R.A. and Feher, G. (1978) Biophys. J. 21, 8a.

[7] Rutherford, A.W. and Evans, M.C.W. (1979) FEBS Lett. 104, 227–230.
[8] Rutherford, A.W. and Evans, M.C.W. (1980) FEBS Lett. 110, 257–261.
[9] Butler, W.F., Calvo, R., Fredkin, D.R., Isaacson, R.A., Okamura, M.Y. and Feher, G. (1984) Biophys. J. 45, 947–973.
[10] Dismukes, G.C., Frank, H.A., Fresner, R. and Sauer, K. (1984) Biochim. Biophys. Acta 764, 253–271.
[11] Debus, R.J., Okamura, M.Y. and Feher, G. (1984) Biophys. J. 45, 255a.
[12] Feher, G., Isaacson, R.A., McElroy, J.D., Ackerson, L.C. and Okamura, M.Y. (1974) Biochim. Biophys. Acta 368, 135–139.
[13] Okamura, M.Y., Abresch, E.C., Isaacson, R.A. and Feher, G. (1984) Biophys. J. 45, 257a.
[14] Nam, H.K., Austin, R.H. and Dismukes, G.C. (1984) Biochim. Biophys. Acta 765, 301–308.
[15] Agalidis, I. and Reiss-Husson, F. (1983) Biochim. Biophys. Acta 724, 340–351.
[16] Cohen-Bazire, G., Sistrom, W. and Stanier, R. (1957) J. Cell. Comp. Physiol. 49, 25–68.
[17] Reiss-Husson, F. and Jolchine, G. (1974) FEBS Lett. 40, 5–8.
[18] Dutton, P.L., Leigh, J.S. and Reed, W. (1973) Biochim. Biophys. Acta 292, 654–664.
[19] Vermeglio, A. (1977) Biochim. Biophys. Acta 459, 516–524.
[20] Rutherford, A.W., Zimmermann, J.-L. and Mathis, P. (1984) in: Advances in Photosynthesis Research (Sybesma, C. ed.) vol.1, pp.445–448.
[21] Rutherford, A.W. (1983) in: The O_2 Evolving System of Photosynthesis (Inoue, Y. et al. eds) pp.63–69, Academic Press, New York.