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Influence of the H-subunit and Fe^{2+} on electron transport from I^- to Q_A in Fe^{2+}-free and/or H-free reaction centers from *Rhodobacter sphaeroides* R-26

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From reaction centres (RC) of *Rhodobacter sphaeroides* R-26 two LM preparations with 0.90 Fe^{2+}/RC (LM) and 0.10 Fe^{2+}/RC (LM/dFe) were prepared. Reconstitution of LM/dFe with the H-subunit and subsequently with Zn^{2+} yielded LMH/dFe and LMH/dFe+Zn preparations, respectively. In these four samples the decay of the primary radical pair P^+I^- was studied by means of transient absorption spectroscopy and compared with that in native RC. In LMH/dFe the reduction of Q_A by Bpheo *a* occurred in 5 ns, with concomitant increase in the yield of P^T, the triplet state of the primary donor. In the LM/dFe, LM and LMH/dFe+Zn preparations the decay of I^- had the same rate (200 ps)$^{-1}$ as in native RC. Thus, neither the H-subunit in the RC nor a divalent metal as Fe^{2+} or Zn^{2+} are necessary *per se* for fast reduction of Q_A. Only demetallation in the presence of the H-subunit slows down the reduction of Q_A.

Reaction center; Quinone; Electron transport; Transition metal; H-subunit; Kinetics; Depletion; Reconstitution; *Rhodobacter sphaeroides*

1. INTRODUCTION

The native reaction center (RC) of *Rhodobacter sphaeroides* consists of three subunits, denoted L, M and H [1]. Photosynthetic charge separation occurs upon excitation of the primary donor P, a dimer of Bchl *a* molecules at the interface of the L and M unit. The L and M subunit each contain one Bchl *a* monomer, one Bpheo *a* and one ubiquinone (UQ_{10}). Q_A is connected to the M subunit, while Q_B is connected to the L subunit. Between the quinones Q_A and Q_B there is a high-spin ferrous ion, interacting with both ubiquinones. The H subunit has no pigment or metal co-factors. At room temperature, Bpheo a_L is reduced in about 3 ps [2]. The reduced Bpheo a_L then transfers the electron to the first quinone acceptor, Q_A, in about 200 ps for native RCs at room temperature [3]. Q_A^- the electron goes to the other ubiquinone, Q_B. When do donors to P^+ and acceptors of $Q_{A,B}^-$ are available, P^+ recombines with one of the semiquinone anions to the singlet state of P. When electron transfer of Bpheo a_L^- to Q_A is slowed down or blocked, the triplet state of P, P^T, is formed with an efficiency that depends on the transfer rate to Q_A [4].

In a previous study on the role of the H-subunit [5], it was concluded that the H-subunit has no effect on the kinetics of electron transfer to Q_A. On the other hand, a study on the role of the transition metal in early electron transfer, especially for the reduction of Q_A, gave contradictory results [6]. Blankenship and Parson [7] and Agalidis et al. [8] observed that the quantum yield of P^+Q^- formation as well as the rate of electron transfer from I^- to Q_A was unperturbed after removal of Fe^{2+}. Kirmaier et al. [9], however, observed that both the quantum yield and the reaction rate were greatly affected by the removal of Fe^{2+}, reconstitution with Zn^{2+}, etc. This discrepancy could be due to a difference in the H-subunit content of the RCs, resulting from differences in the preparation procedure. We have therefore re-examined the role of the H-subunit and the divalent metal in early electron transport in Fe-free and/or H-free RCs. To this end we have prepared RCs from which either Fe^{2+} or the H-subunit, or both were removed. The decay of the primary radical pair P^+I^- in differently modified and native RCs was studied by transient absorption difference spectroscopy with picosecond time resolution. Comparison of the results obtained with the above-mentioned preparations indicates that both the H-subunit and the ferrous ion play an essential role in electron transport from I^- to Q_A.

Abbreviations: Q_A and Q_B, primary and secondary electron acceptors; Vitamin K_1, 2-methyl-3-phytyl-1,4-naphthoquinone; UQ, ubiquinone; Bchl *a*, bacteriochlorophyll *a*; Bph *a*, bacteriopheophytin *a*; LDAO, lauryldimethylamine *N*-oxide; EDTA, ethylenediaminetetraacetic acid; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis.

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2. MATERIALS AND METHODS

2.1. *Materials*

LDAO was purchased from Fluka Chemical Corporation; UQ_{10} was obtained from Sigma; Sodium cholate, EDTA and 1,10-*o*-phenanthroline monohydrate were from Merck; DE-52 was from Whatman; $LiClO_4$ was from BDH. UQ_{10} was dissolved in sodium desoxycholate solution by the procedure described previously [10], except that the quinone was first dissolved in diethyl ether instead of ethanol.

2.2. *Reaction centers*

Native RCs from *Rb. sphaeroides* R-26 were prepared using the detergent LDAO essentially as described in the literature [11] with the following modifications: The optical density of the chromatophore suspension at the maximum intensity was adjusted to 60 (1-cm path length) instead of 40, and 1.2% LDAO instead of 1% LDAO was added to solubilize the RCs. The 'crude' RCs were then purified by two times ammonium sulfate precipitation at 0°C instead of at room temperature (30% and 28% w/v ammonium sulfate was used for the first and the second precipitation, respectively). The concentration of LDAO was lowered to 0.3% before the ammonium sulfate precipitation to minimize the loss of Q_B. Following the second precipitation, the RCs were precipitated on Celite and washed first with 26% w/v ammonium sulfate in TL buffer (10 mM Tris-HCl, 0.1% LDAO, 0.1 mM EDTA, pH 8.0, 4°C), then with 23% w/v ammonium sulfate in TL buffer, and finally eluted with 18% w/v ammonium sulfate in TL buffer. After deslating on a G-50 column, the RCs were further purified on a DE-52 column by washing with TL buffer overnight and then eluted with a salt gradient (100 ml, 50–175 mM). RCs with an absorbance ratio A_{280}/A_{803} of 1.20 were collected and dialyzed against 10 mM Tris-HCl, 0.025% LDAO and 0.1 mM EDTA, pH 8.0, 4°C, and concentrated using an Amicon Ultrafiltrater with a 30 kDa membrane filter at 0°C. RCs prepared in this way typically contained 0.90 Fe/RC as determined by atomic absorption spectroscopy (see Fe-analysis), and ~1.85 Q/RC as determined spectrophotometrically [12].

2.3. *LM complex with Fe^{2+} (LM) and LM complex without Fe^{2+} (LM/dFe)*

The isolation of LM complex with Fe^{2+} (LM) was based on the procedures described previously [6,9]. RCs in 10 mM Tris-HCl, 0.025% LDAO, 0.1 mM EDTA ($8 < A_{803}^{1cm} < 14$) were dialyzed for 1 h at room temperature against a buffer of 10 mM Tris-HCl, 0.025% LDAO, pH 8.0, containing 0.75 M $LiClO_4$, 50 mM $CaCl_2$ and 10% v/v ethanol. After centrifugation (5000×*g*, 10 min, 4°C), the precipitated H-subunit was discarded and the supernatant was immediately dialyzed for 1 day at 4°C against 10 mM Tris-HCl, 0.025% sodium cholate and 0.1 mM EDTA, pH 8.0, with several changes of the buffer. A solution of about 2 mM UQ_{10} in 10% sodium deoxycholate (5% v/v) prepared as described above was added prior to dialysis. The LM complex without Fe^{2+} (LM/dFe) was similarly prepared, except that 1 mM *o*-phenanthroline was added to the dialysis solution. The purity of the preparations was checked by the A_{280}/A_{803} ratio, which was lowered from 1.2 to 1.0 in both preparations as a result of the deletion of the non-pigmented H-subunit. The concentration of the LM/dFe preparation was determined using for extinction coefficient at 803 ε_{803} = 288 $mM^{-1}\cdot cm^{-1}$, as determined previously [5].

2.4. *Reconstitution of the LM/dFe preparation with the H-subunit (LMH/dFe)*

H-subunits were isolated from native RCs by the method of Agalidis et al. [8], except for using sodium cholate instead of Deriphat 160 and pH 7.7 (4°C) instead of pH 8.0. The concentration of the H-subunit was determined using the extinction coefficient ε_{280}=46 $mM^{-1}\cdot cm^{-1}$ [5]. Reconstitution of the LM/dFe preparation with the H-subunit was performed by mixing the H-subunit (freshly made) and LM/dFe complex at an approximate molar ratio of 1.8H/LM at 0°C. After adding a solution of ~2 mM UQ_{10} in 10% sodium desoxycholate prepared as described above, the mixture was then dialyzed against 10 mM Tris-HCl, 0.025% sodium cholate and 0.1 M EDTA, pH 7.7 for 2 days at 4°C with several changes of the buffer. The LMH/dFe preparation was purified by chromatography on a De-52 column or on an organomercurial Agarose (Affi-Gel 501, Bio-Rad) column as previously described [10].

2.5. *Reconstitution of the LMH/dFe preparation with Zn^{2+} (LMH/dFe+Zn)*

Fe-depleted RCs were reconstituted with Zn^{2+} using the method of [10] with minor modifications. The LMH/dFe preparation prepared as described above (A_{803}^{1cm}=10 in 30 mM Tris-HCl, 0.025% sodium cholate, and 0.1 mM EDTA, pH 7.7) was incubated with 2 mM $ZnCl_2$ for 1 day at 4°C and then dialyzed for 2 days against 10 mM Tris-HCl, 0.025% sodium cholate and 1 mM EDTA, pH 7.7 with several changes of the buffer.

2.6. *Fe-analysis*

The Fe^{2+} content was determined by Atomic Absorption spectroscopy, using a Perkin-Elmer 460 Atomic Absorption spectrophotometer with flame. The standard solution was made by diluting 5 ppm Fe^{2+} in 3% HNO_3, with a buffer of 10 mM Tris-HCl, 0.025% sodium cholate and 0.1 mM EDTA, pH 7.7, the same buffer as that of the sample. The blank solution (buffer only) contained 0-0.025 ppm Fe^{2+} representing a background level of < 0.01 Fe/RC. The accuracy of the Fe determination was 0.02 Fe/RC. As a check the presence of Fe^{2+} was monitored by recording the Fe^{2+}-Q^- EPR signal of RCs frozen under illumination.

2.7. *Gel electrophoresis*

The purity of different preparations was assayed by both SDS-PAGE and Agarose gel electrophoresis under non-denaturing conditions. The SDS-PAGE was performed as described previously [13]. Agarose gel electrophoresis under non-denaturing conditions was performed as previously reported by Debus et al. [10], using as controls native RCs. The buffer used in the gel, the samples and the electrode reservoirs were the same as described by Debus [10]: 50 mM Tris-HCl, 0.1% LDAO, 0.025% sodium cholate and 1 mM EDTA (pH 8.0). 2 mm thick gels containing 1.2% agarose (FMC Corp.) were cast on Gel bond film (FMC Corp., 7.5×5.0 cm). ~7 μl sample (A_{803}^{1cm}=10 in 10 mM Tris-HCl, 0.1% LDAO, 0.025% sodium cholate, 0.1 mM EDTA and 10% sucrose, pH 8.0) were applied and electrophoresed at 4°C and 10 V/cm for 3 h. The gel was pre-electrophoresed under the same conditions for 30 min before applying the sample. Following electrophoresis, the gels were fixed and stained with Coomassie blue and air-dried at room temperature.

2.8. *Photochemical activity*

The photochemical activity defined as the formation of P^+Q^- was determined optically by monitoring the light-induced absorbance change at 865 nm as described elsewhere [14].

2.9. *Decay kinetics of P^+I^-*

Absorbance differences due to the Bpheo *a* anion were measured at 668 nm [9] using a pump-probe method. For excitation we used the frequency-doubled 532 nm pulse of a passively mode-locked Nd:YAG laser (JK Lasers). The duration of this excitation pulse was about 25 ps. A weak probe pulse was generated by passing the remaining 30 ps, 1064 nm fundamental pulse through a cuvette filled with a H_2O/D_2O mixture. From the resulting continuum ('white') pulse the desired probe pulse was extracted by means of a small monochromator with a band width of about 1 nm. The instrumental response, resulting from the cross-correlation of pump and probe pulses, was approximated by a gaussian of 35 ps FWHM. Fits to the measured kinetics were convolutions of exponential decays with this instrument's response function. An additional parameter used in the fit was the triplet-minus-singlet absorbance difference remaining after decay of I^-, which was taken to be constant on the timescale of the experiment. A sample for measurement was prepared by thawing a stock solution and adding glycerol. The final content was 50–66%. *o*-Phenantroline was added to block Q_B reduction, thus providing total P^+ recombination between successive excitation flashes. The solution was cooled in a home-made cryostat with a water–alcohol mixture as cooling liquid. Temperature were between −2 and 0°C.

3. RESULTS

3.1. *Characteristics of different modified preparations*

Table I summarizes some characteristics of the different preparations. The extraction and reconstitution of the transition metal has no effect on the optical absorption ratio A_{280}/A_{802}. On the other hand, this ratio was decreased from 1.2 to 1.0 when the H-subunit was removed. Reconstitution with the H-subunit resulted in the recovery of the ratio of 1.2. Under non-reducing conditions the triplet EPR signal of P^T was only observed in the LMH/dFe preparation, which has no Fe^{2+} but still retains the H-subunit. Also, only such a preparation showed a considerable decrease of the yield of P^+Q^- (65%).

The purity of the different preparations was checked by electrophoresis both under denaturing and non-denaturing conditions. LM and LM/dFe preparations showed only L and M bands in the SDS-PAGE, and only the LM band in the non-denaturing agarose gel electrophoresis. These observations indicate that in both preparations the H-subunit was removed, in agreement with data of Debus et al. [5], who found that the LM/dFe preparation contained less than 5% remaining H-subunit. The LM complex contained 0.90 Fe/RC and the LM/dFe complex contained 0.10 Fe/RC as determined by atomic absorption spectroscopy assay. The purity of the LMH/dFe preparation was checked by both SDS-PAGE and agarose gel electrophoresis under non-denaturing condition. Three bands corresponding to L, M and H were observed in SDS-PAGE and only one band corresponding to native RCs was observed in agarose gel electrophoresis. In addition, reconstitution of the H-subunit led to the recovery of the 1.2 absorption ratio, the formation of P^T under non-reducing conditions and the decrease of the yield of P^+Q^- (65%). These observations prove that the reconstitution of the H-subunit to a level > 95% has been successful.

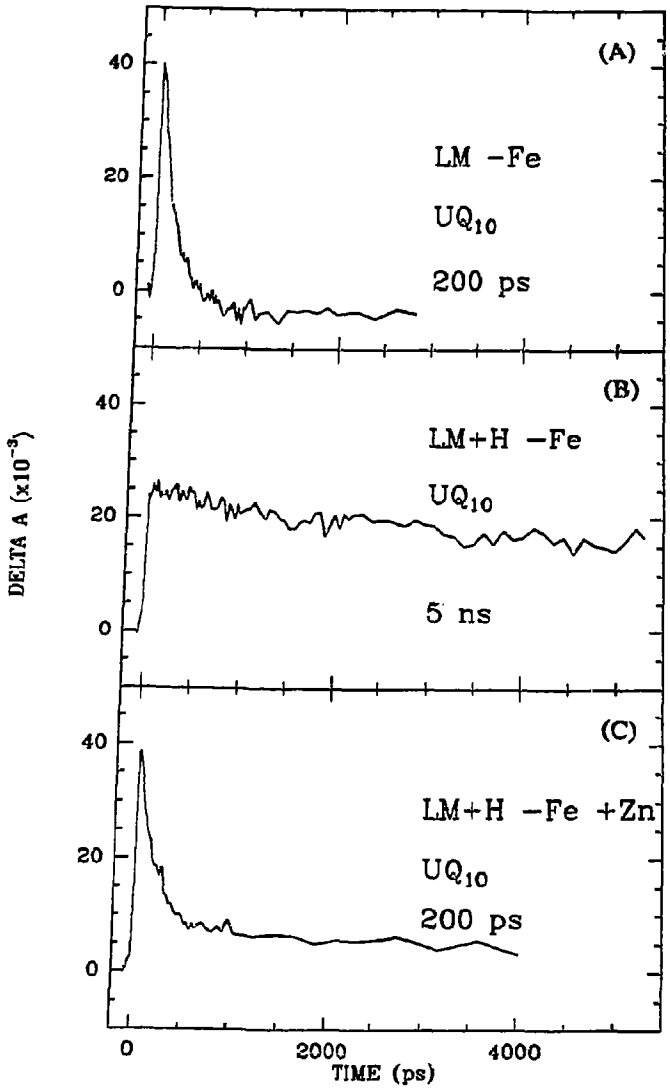

Fig. 1. Kinetics of the absorbance change at 668 nm of (A) Lm/dFe, (B) LMH/dFe and (C) LMH/dFe+Zn preparations from reaction centers of *Rb. sphaeroides* R-26. For excitation we used the frequency-doubled 532 nm pulse of a passively mode-locked Nd:YAG laser (JK Lasers). The duration of this excitation pulse was about 25 ps.

Table I

Some characteristics of different preparations

Prep*.	Spectra A_{280}/A_{803}	EPR Fe-Q^-	EPR P^T	Subunit composition SDS-PAGE L	SDS-PAGE M	SDS-PAGE H	Agarose gel H	Agarose gel LM	Agarose gel LMH	Fe-content Fe/RC	*Activity %	Decay time of I^- (ps)
LMH	1.2	+	−	+	+	+	−	−	+	0.91	100	200
LM	1.0	+	−	+	+	−	−	+	−	0.91	95	200
LM/dFe	1.0	−	−	+	+	−	−	+	−	0.10	95	200
LMH/dFe	1.2	−	+	+	+	+	−	−	+	0.10	65	5000
LMH/dFe+Zn	1.2	−	−	+	+	+	−	−	+	0.10	85	200

*The activity defined as the formation of P^+Q^- was determined by monitoring the light-induced absorbance change at 865 nm.

3.2. *Rate of Electron Transfer from I^- to Q_A*

The electron transfer from I^- to Q_A in different modified and native RCs was measured by monitoring the decay of I^- with transient absorption difference spectroscopy with picosecond time resolution. Fig. 1 shows the kinetics measured at 668 nm. At this wavelength a positive ΔA occurs in the Bpheo *a*–Bpheo *a* spectrum. The first trace in Fig. 1A shows the kinetics of a LM/dFe preparation containing 0.10 Fe/RC and <5% H-subunit. The 668 nm ΔA recovers fast, a best fit yielding a decay time of 200 ps, and the remaining constant signal is very small, even negative in this case. These results are virtually indistinguishable from those measured for the native RCs (data not shown), indicating that in the LM/dFe preparation forward electron transfer from the Bpheo *a* anion to Q_A is fast with negligible triplet yield. The second trace in Fig. 1B shows the signal obtained with a purified LMH/dFe preparation containing < 0.10 Fe^{2+} per RC that has been reconstituted with the H-subunit. The decay at 668 nm is now much slower than in Fig. 1A. The triplet yield is considerable, as indicated by the large positive constant component. A best fit to the data yielded a decay time of 5 ns, and a triplet contribution of 30%.

Reconstitution of LMH/dFe with Zn^{2+} was performed to check whether the change observed in the LMH/dFe preparation was caused by the reconstitution of the H-subunit as such, or by a possible effect of the reconstitution procedure. Fig. 1C shows that upon reconstitution of the LMH/dFe preparation with Zn^{2+} both the fast decay rate of I^- and the high yield of P^+Q^- recovered, a best fit yielding a decay time of 200 ps. In addition the triplet EPR signal of P_T under non-reducing conditions largely disappeared, the remaining signal at 4 ns (about 15%) being due to 'inactive' RC (Table I), which lack a functional quinone acceptor. These results show conclusively that the differences in electron transport of the LM/dFe and the LMH/dFe preparations are due to the absence and the presence of the H-subunit, respectively.

4. DISCUSSION AND CONCLUSIONS

4.1. *Effect of the H-subunit and the Fe^{2+} Ion on the Electron Transfer from I^- to Q_A*

The two major findings of the present are that (i) the absence or presence of Fe^{2+} has no effect on the early electron transfer to Q_A when the H-subunit is absent, and (ii) the absence of the H-subunit has no effect on the early electron transfer when Fe^{2+} is present. Thus, both the H-subunit and Fe^{2+} are not essential for early electron transport in bacterial RCs when considered separately.

There is, however a subtle interplay between Fe^{2+} and the H-subunit. The reconstituted LMH/dFe preparation and a preparation in which only the Fe^{2+} was removed (RC/dFe, data not shown) both showed a slow electron transfer rate from I^- to Q_A $(\sim ns)^{-1}$ with concomitant low yield of P^+Q^-, and a triplet EPR signal under non-reducing conditions. Similar results were obtained by Kirmaier et al. [9]. Reconstitution of a LMH/dFe preparation with Zn^{2+} resulted in the recovery of the fast forward electron transfer from I^- to Q_A and high yield of P^+Q^-, in agreement with the results observed for RC/Zn by Debus et al. [10] and Kirmaier et al. [9], and the disapperence of the triplet EPR signal under non-reducing conditions. Thus, the influence of Fe^{2+} on early electron transport can only be observed in RCs when the H-subunit is still associated to it, and as a counterpoint, the influence of the H-subunit can only be observed in RCs from which the transition metal is removed. In other words, early electron transport is only slowed when the Fe^{2+} is removed and the H-subunit is present. In all other cases, viz. both Fe^{2+} or another divalent cation as Zn^{2+} and the H-subunit present, or both absent or only the H-subunit removed, early electron transport is unimpaired.

The above findings allow to solve a discrepancy reported in the literature [6]. The conclusion drawn by Debus et al. [5] that the H-subunit does not play an essential role, was based solely on observations on preparations in which the transition metal was still present. Therefore their conclusion was only partly true. It is now clear that the H-subunit does play a vital role in the early electron transport in preparations in which the transition metal ion is absent. Similarly, the conclusion by Kirmaier et al. [9] that a divalent metal ion is essential for unimpaired electron transport was based on a preparation with intact three-subunit structure. Our results clearly show that in LM particles the presence of a divalent metal ion is not necessary for unimpaired early electron transfer.

The fast forward electron transfer from I^- to Q_A and high yield of P^+Q^- observed by Blankenship and Parson [7] in preparations with much less than stoichiometric amounts of Fe^{2+} can be attributed to their RCs not having retained the H-subunit. It is now known that the $KClO_4$ used in their procedure removes not only Fe^{2+} but also the H-subunit [10]. So the fast forward electron transfer rate they observed in fact resulted from the LM/dFe complex instead of the RC/dFe complex.

The 450 ps electron transfer rate and 90% yield of P^+Q^- observed by Agalidis et al. [8] can be explained by the following contributing factors. Firstly, it is likely that their reconstitution of the H-subunit was not complete, the H-subunit reported to be only weakly associated to the LM unit in their preparation [8]. Then an intermediate state between the LM/dFe complex and RC/dFe is observed, yielding an intermediate electron transfer rate (between $(200\ ps)^{-1}$) and $(5ns)^{-1}$). The other factor is that they used UQ_6 instead of native UQ_{10} for the reconstitution of Q_A. We have measured the electron transfer rate from I^- to Q_A in an LMH/dFe preparation in which the native UQ_{10} was replaced either by UQ_6 or

by vitamin K_1 (data not shown). UQ_6 gave a rate of $(\sim 1\ ns)^{-1}$, vitamin K_1 $(200\ ps)^{-1}$. These results indicate that the nature of the primary acceptor plays a vital role on the early electron transport in RCs also in the absence of the transition metal. Work on the influence of different quinones on the early electron transport in the Fe-free and/or H-free RCs is currently in progress.

4.2. *A possible explanation of the influence of the divalent metal and the H-subunit on electron transport from I^- to Q_A*

Our results demonstrate that the absence of a divalent metal per se does not cause a slowing down of the rate of reduction of Q_A. This means that the divalent metal does not change the in situ redox potential as has been suggested [9], as it would be highly unlikely that the removal of the H-subunit would exactly compensate the purported redox potential shift. More likely, the slowing down of early electron transport is a result of structural changes wrought by the absence of the metal. These structural changes are apparently undone when the H-subunit is removed from the metal-less reaction center. Thus, it seems that two seemingly unrelated actions, namely the removal of the metal and the removal of the H-subunit, have precisely opposite effects. The structural change invoked above must therefore be quite specific. We believe that this change corresponds to a repositioning of the quinone molecule from one well-defined position to another. It has been reported [15] that the Q_A molecule can occupy two slightly different sites, the normal A position and a sterically equally unhindered B position, which is shifted laterally with respect to the $Bphe_A$ molecule by about half a quinone diameter (see Fig. 1 of Ref. 15, schematically depicted in Fig. 2a). Thus, we propose that removal of the divalent metal switches the quinone from the native A position to the B position. Subsequent removal of the H-subunit then switches the quinone back to its normal A position. The change in quinone position should be reversible, as observed. When the Q_A molecule occupies the native A position, electron transfer rate from I^- to Q_A is unimpaired. If, however, the Q_A molecule moves to the B position, this rate slows down considerably.

The switching effect of the divalent metal likely results from the indirect interaction of Q_A with the metal via histidine. The switching effect of the H-subunit is probably mediated by the interaction of its transmembrane helix with the Q_A site.

When both Fe^{2+} and the H-subunit are absent, electron transport from I^- to Q_A is not impaired. Thus, in this preparation the quinone occupies the native A position.

We can depict the proposed mechanism by two 'springs' connecting Fe^{2+} ('Fe-spring') and the H-subunit ('H-spring') to Q_A (Fig. 2). The 'springs' represent the above-mentioned interactions, whose precise nature remains to be specified. The action of the two 'springs' is opposite to each other. The action of the 'Fe-spring' is stronger than that of the 'H-spring', keeping Q_A in the native A position (see Fig. 2a). If Fe^{2+} is removed (RC/dFe), the 'H-spring' moves Q_A to the B position (see Fig. 2b). When H is subsequently removed (LM/dFe), Q_A moves back to the normal A position because of the absence of the 'H-spring' (see Fig. 2d). Reconstitution of LM/dFe with H moves Q_A again to the B position. In the LM/Fe preparation the 'Fe-spring' keeps Q_A in the normal A position (see Fig. 2c).

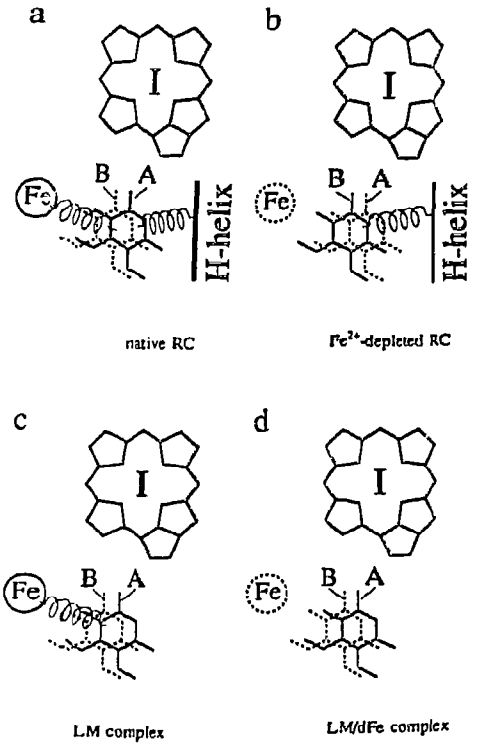

Fig. 2. A proposed model for the influence of the divalent metal and the H-subunit on electron transport from I^- to Q_A in different reaction center preparations from *Rb. sphaeroides* R-26. a, Native RC (LMH); b, Fe-depleted RC (LMH/dFe); c, LM complex with Fe^{2+} (LM); d, LM complex without Fe^{2+} (LM/dFe).

The above explanation, involving different positions of Q_A for the different preparations, can be verified by X-ray analysis of single crystals of divalent-metal-less RC. This work is currently in progress in the laboratory of Dr G. Feher, University of California at San Diego, USA (private communication).

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