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Crystal Structure of the *Rb. sphaeroides* Zn^{2+}-binding Reaction Centre Mutant HC(M266).

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Introduction

In bacterial RCs light-induced electron transfer proceeds from the primary donor, a bacteriochlorophyll dimer, to the first (Q_A) and secondary (Q_B) quinone. As both Q_A and Q_B in the *Rb. sphaeroides* reaction centre (RC) are ubiquinone-10 molecules their different properties in the electron transfer chain must be the result of interactions with the protein environment. This can be probed by EPR techniques performed on the semiquinone radical anions (for review see [Lubitz & Feher 1999]). However, application of EPR is hindered due to the presence of the high-spin Fe^{2+} that interacts magnetically with these radicals. Here, replacement of the Fe^{2+} by diamagnetic Zn^{2+} [Debus 1986] is possible but the resulting $ZnRC_c$ are very hard to crystallize. It has been shown, however, that a mutant, HC(M266), in which one of the His ligands of the iron is replaced by Cys, incorporates Zn^{2+} from the growth medium [Williams 1991] yielding RCs with >90% Zn incorporation ($ZnRC_m$). In this communication we report on the crystallization of $ZnRC_m$ from the mutant HC(M266). The structure was solved to 2.6 Å resolution and compared to that of the wild type FeRC. The data are supplemented by FTIR difference ($Q_A^{-\bullet}/Q_A$) spectroscopy and EPR/ENDOR studies of $Q_A^{-\bullet}$.

Materials and Methods

Crystals were grown using the sitting drop method containing potassium phosphate as precipitant, and heptanetriol, hexanetriol and dioxane as additives [Buchanan et al, 1993]. The crystals belong to the trigonal space group $P3_121$ (a=b=141.8Å, c=187.2Å) and diffracted X-rays to 2.6Å resolution. The data was collected on a single crystal at the EMBL Outstation BW7B synchrotron beamline (DORIS ring/DESY-Hamburg, Germany), processed with the

HKL software package and refined using CNS 0.3 [Kuglstatter, 1999]. Simulated annealing omit maps were calculated to determine the structure around Zn^{2+} and Q_A without model bias. ENDOR spectra were obtained as described [Lubitz and Feher, 1999 and references therein]. FTIR was performed essentially as in [Breton et al, 1994] except that stigmatellin was used as the Q_B inhibitor.

Results and Discussion

The structure of ZnRC HC(M266) (called $ZnRC_m$) has been solved to 2.6Å resolution. Figure 1 shows the structure in the vicinity of the Zn^{2+} co-ordinating atoms and the Q_A. pocket with all the relevant distances presented in Table 1.

Table 1. Distances (in Å) from the metal to the closest heavy atom of the respective amino acid side chains in the wild type FeRC (Brookhaven data bank entry 1AIJ) and $ZnRC_m$ All residues act as ligands except the mutated cysteine which in the mutant structure is too distant. Also shown is a comparison of the respective distances from the carbonyl oxygens of Q_A to the nitrogen atoms of the given residue involved in H-bond formation (na = not applicable).

	His M219	***His L130***	***His L190***	***Glu M234***	***His M266***	***Cys M266***	***O4-Nδ His M219***	***O1-Ala M260***
FeRC	2.2	2.3	2.1	2.2 & 2.2	2.1	na	2.8	2.8
$ZnRC_m$	2.1	2.1	2.0	2.2 & 2.3	na	5.0	2.8	2.8

The Zn^{2+}-Cys M266 distance is 5.0Å compared to the 2.1Å Fe^{2+}-His M266 distance in the wild type RC. Thus, cysteine no longer acts as a ligand to the metal. The altered geometrical and electronic environment in the non-heme iron site could provide an explanation for the preferential incorporation of zinc rather than iron into this site. The second quinone (Q_B) occupies the dark-adapted 'distal' site (Q_{B1}) reported in [Stowell *et al*, 1997] with O1 of Q_B 6.6Å away from Nδ of His L190.

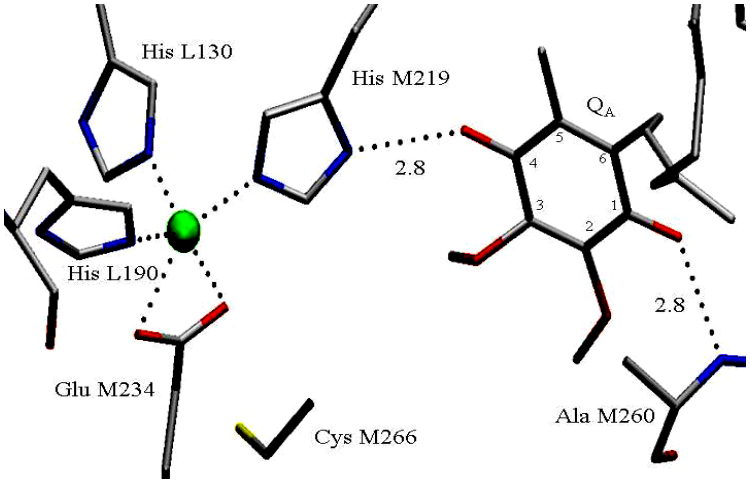

Figure1. Structure of $ZnRC_m$ in the vicinity around the zinc co-ordinating atoms and the Q_A pocket. The Zn^{2+} is given as the green sphere ligated to three histidines and one glutamate (bidentate) ligand. The putative H-bonds formed by the carbonyl oxygens of the quinone are given as dotted lines and their respective lengths are indicated.

Crystallographically there are no significant differences apparent at this resolution (error ~ 0.25 Å) between $ZnRC_m$ and wild type FeRC.

Figure 2 presents $Q_A^{-\bullet}/Q_A$ FTIR difference spectra for FeRC and $ZnRC_m$.There are some differences in the bonding interactions of $Q_A^{-\bullet}$ in $ZnRC_m$ compared to the wild type FeRC. The largest effect is seen on the coupled C⋯O and C⋯C mode of $Q_A^{-\bullet}$ peaking at 1464 cm^{-1} in the wild type. This is altered in the mutant where it downshifts to 1443 cm^{-1}. On the other hand, the predominantly C⋯C modes at 1485 and 1421 cm^{-1} are little affected by the mutation. The IR modes of the neutral Q_A molecule appear only slightly perturbed with the C_4=O mode at 1601 cm^{-1} and the C=C mode at 1628 cm^{-1} in wild type being downshifted in the mutant by only 2 and 4 cm^{-1}, respectively. The IR contribution of the protein vibrations, notably at 1670 and around 1550 cm^{-1}, and of the bacteriopheophytin H_A modes responsible for the signals above 1700 cm^{-1} are essentially unaffected upon mutation. The $Q_A^{-\bullet}/Q_A$ FTIR difference spectrum of chemically exchanged $ZnRC_c$ is very similar to that of FeRC. This indicates that in $ZnRC_m$ the His→Cys replacement causes a slight change in the bonding between $Q_A^{-\bullet}$ and the surrounding amino acids.

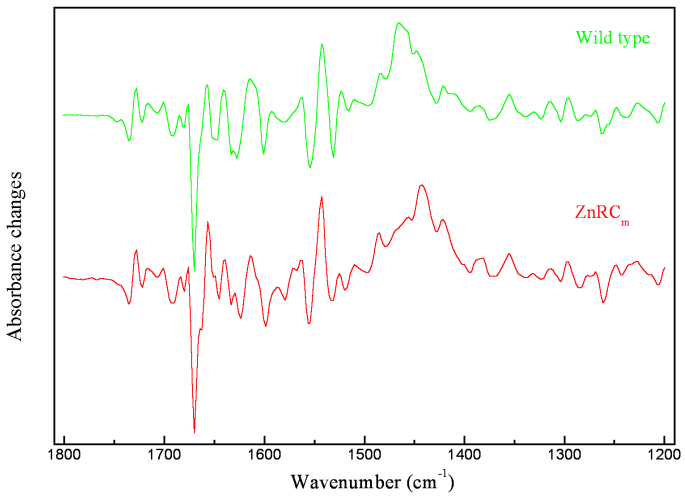

Figure 2: Light-induced $Q_A^{-\bullet}/Q_A$ FTIR difference spectra of wild type and $ZnRC_m$ of *Rb. sphaeroides* recorded at 5°C in the presence of stigmatellin. The maximum peak-to-peak amplitude is close to 10^{-3} absorbance units.

To further investigate the difference between $ZnRC_c$ and $ZnRC_m$ EPR and ENDOR spectra have been taken of $Q_A^{-\bullet}$ from both systems, cf. also [Lubitz and Feher 1999]. Some hyperfine couplings are given in Table 2 showing shifts of the spin density distribution. The H-bond couplings are somewhat weaker in $ZnRC_m$ indicating a 'looser' binding site in the mutant. In both ZnRC the H-bond between His and O_4 of $Q_A^{-\bullet}$ is shorter than the one between Ala and O_1 (see Fig. 1), which is deduced from the direction of the spin density shift in $Q_A^{-\bullet}$ [Lubitz and Feher 1999]. Application of the simple point-dipole model, using an oxygen spin density of 0.2, indicates a difference in distance between the two H bonds in $ZnRC_c$ of only ~0.15 Å and in $ZnRC_m$ of 0.17 Å. The somewhat more asymmetric H bond situation in the mutant is supported by the increased difference between the proton couplings at pos. 5 (CH_3) and pos. 6 (CH_2) of $Q_A^{-\bullet}$ in $ZnRC_m$ (Table 2). These subtle differences detected by magnetic resonance methods cannot be obtained from the X-ray structure at the present resolution.

Table 2: Isotropic hyperfine components [MHz] of the methyl and methylene groups of $Q_A^{-\bullet}$ in $ZnRC_m$ compared with $ZnRC_c$ (T = 253 K). For the exchangable protons of the H-bond only the pronounced perpendicular components of the tensor are given.

position	***hfc***	***ZnRC c***	***ZnRC m***
5 (CH_3)	A_{iso}	4.9	4.6
6 (CH_2)	A_{iso}	6.2	6.5
O_1 H-bond	$A_\perp$	4.2	4.0
O_4 H-bond	$A_\perp$	5.7	5.6

Conclusion

Although at the present resolution of the X-ray crystallographic structure no significant changes of the quinone binding sites could be detected in $ZnRC_m$ and FeRC FTIR difference spectroscopy indicates a slightly different Q_A binding situation. Changes are also obtained from evaluation of the hfcs of $Q_A^{-\bullet}$ from ENDOR spectroscopy on ZnRC of the wild type and the mutant. In conclusion it can be stated that care must be taken when quantitative conclusions are drawn about the system from samples of the HC(M266) mutant. This is certainly a general problem when data from mutant RCs are used to explain structural and functional details of the native system although often a qualitatively correct picture can be obtained.

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