Influence of Cd$^{2+}$ on the spin state of non-heme iron and on protein local motions in reactions centers from purple photosynthetic bacterium *Rhodospirillum rubrum*.

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**Abstract.** Non-heme Fe is a conservative component of the Q-type photosynthetic reaction centers but its function remains unknown. Applying Mössbauer spectroscopy we show that in *Rhodospirillum rubrum* the non-heme Fe exists mostly in a ferrous low spin state. The binding of Cd$^{2+}$ ions in the vicinity of the quinone-Fe complex changes the high spin state of the non-heme Fe into a low spin one characterized by hyperfine parameters similar to those obtained for the non-heme Fe low spin state in untreated reaction centers, as confirmed by Mössbauer measurements. The nuclear inelastic scattering of synchrotron radiation experiments show that the contribution of vibrations at low energies, between 3-15 meV, activated at 240 K are damped in the bacterial reaction centers treated with CdCl$_2$. No influence of Cd$^{2+}$ ions is observed on the soft vibrational states at 60 K. These results suggest that binding of cadmium cations within the reaction centers may enhance decoupling of the non-heme Fe from the surrounding protein matrix at temperatures higher than 200 K, what can explain the slowing down of electron transfer between the Q$_A$ and Q$_B$ quinones by Cd$^{2+}$.

1. Introduction

Photosynthesis is the ultimate source of energy for all living organisms on our planet, which relies on the conversion of solar energy into biologically-useful chemical energy by specialized pigment-protein complexes called photosynthetic reaction centers (RCs). Depending on the type of the final electron...
acceptor in the electron transport (ET) chain, RCs are classified into two types: Q- and Fe-S type. Bacterial Q-type RCs belong to the best recognized photosynthetic structures. Due to their structural simplicity they serve as a model system in studies of intramolecular electron transfer (ET) within proteins [1, 2]. The core of bacterial RC is composed of two peptides L and M binding several cofactors arranged in two symmetric branches: a dimer of two bacteriochlorophylls called a special pair (BP870A and BP870B), two accessory bacteriochlorophylls (Bα and Bβ) in proximity to the special pair, two bacteriopheophytins (BPheA and BPheB), a pair of ubiquinones (UQA and UQB) with a non-heme iron (NHFe) located between them (Fig.1A). It is believed that in native systems only left branch is active in linear electron transfer and the direction of electron flow from BP870 to the final quinone acceptor at site QB is marked by arrows in Fig.1A. When ubiquinone at the QB site is doubly reduced it accepts two H+. Then, the ubiquinol form, UQH2, is exchanged by the external oxidized UQ. The whole process is light driven by excitation of BP870, which is a primary electron donor. Its oxidized form is reduced by cytochrome c2.

**Figure 1.** Scheme of the core proteins M and L of bacterial reaction center with redox active cofactors participating in linear ET (A). The black arrows show the subsequent steps of ET and the white arrow indicates the site excited by light. In (B), an arrangement of aminoacids from the proteins: M, L and H in the vicinity of the NHFe binding site for *Rs. rubrum* RC is shown [19, 20]. The aminoacids of the H protein are taken from Rb. sphaeroides. Symbols: BP870 – bacteriochlorophyll dimer (special pair); Bα, Bβ – bacteriochlorophyll monomers; BPheA, B – bacteriopheophytin; UQA, UQB – ubiquinone, UQ – oxidized form of ubiquinone, UQH2 – ubiquinol form, NHFe – non-heme iron.

The efficiency, the rate of primary charge separation and its pathways in RCs critically depend on the distances, specific arrangement and electronic properties of the ET cofactors, which in turn are strongly determined by protein matrix. The molecular mechanisms regulating photosynthetic ET have long been a subject of intensive studies but because of their complexity it is still unclear how exactly the protein matrix exerts its control over the ET cofactors [3-6]. In particular, the role of NHFe, a very conservative component of the Q-type RCs, in photosynthetic charge separation and the temperature effects on ET between the two external quinone acceptors are among the most challenging issues [7, 8]. Intriguingly, ET from reduced QA to QB is inhibited at temperatures below 200±20 K whereas QA in RCs from various organisms remains fully active at cryogenic temperatures [9, 10]. This is in line with the theoretical calculations which suggest that some intrinsic flexibility of the protein matrix in the vicinity of the QA–Fe–QB complex is required for efficient ET on the RCs acceptor side [11]. The collective motions of the protein matrix in RCs, activated above 180 K seem to be involved in the activation of ET within the QA–Fe–QB complex, along with the protonation and deprotonation events on the acceptor side of RCs [12-14]. However, the role of NHFe in stabilization of the QA and QB binding sites and in the primary ET is still under debate because a change of the
NHFe valence state has never been detected in native systems [15]. Such changes were observed only in RCs in algae PSI mutant [16, 17].

In this paper we show that NHFe in RC isolated from *Rhodospirillum (Rs.) rubrum* occurs almost exclusively in a low spin state, while in RC from *Rhodobacter sphaeroides* it was observed in a high spin state [7, 18]. At the same time, the rate of ET in the former RC is slower [19]. We suggest that this effects may be related to the differences of the amino acid sequences in the NHFe surrounding between these two types of bacterial RCs (see Fig. 1B, [20,21]). These intriguing observations prompted us to investigate in detail NHFe in *Rs. rubrum*, in particular, how its low spin state is affected by fluctuations of protein matrix and by Cd$^{2+}$ ions, which are known to bind in the vicinity of the iron-quinone complex [22, 23]. To this end, Mössbauer spectroscopy was applied to compare the valence and spin states of Fe atoms in native RC from *Rs. rubrum* and in RC treated with Cd$^{2+}$ salt. Further, nuclear inelastic scattering (NIS) was used to monitor the collective motions in the NHFe-binding sites in both the native and the Cd$^{2+}$-treated RCs.

2. Materials and Methods

*Chromatophores preparation and isolation of reaction centers.* The wild type bacterium, *Rs. rubrum* S1 was grown under anaerobic conditions in the white light at 27°C in a modified Hutner medium [24] supplemented with $^{57}$Fe. The enrichment of the chromatophores in $^{57}$Fe was 30-50% as estimated by the use of atomic absorption (ICP-MS ELAN 6100 spectrometer, Perkin Elmer) and Mössbauer spectroscopy. The RCs were prepared according to a described method with some modifications [25, 26]. The final purification step was done by ion-exchange chromatography on DEAE-Cellulose (DE52 Whatman) in the presence of 0.08% LDAO. The purified RCs were stored at -40°C. The quality of the RC preparation was assessed by absorption spectroscopy (Fig. 2; Cary 50 Bio spectrophotometer, Varian USA) and confirmed in the test for the photochemical activity (data not shown).

**Figure 2.** Absorption spectrum of *Rs. rubrum* reaction center. Symbols: BP$_{870}$ – bacteriochlorophyll dimer (special pair); BChl$_{A,B}$ – bacteriochlorophyll monomers; BPhe$_{A,B}$ – bacteriopheophytin; Crt- carotenoid.

*Mössbauer spectroscopy.* The Mössbauer $^{57}$Fe spectra were recorded in a home made cryostat using 50 mCi $^{57}$Co/Rh as a source of 14.4 keV γ radiation and a proportional counter to detect the radiation. The temperature stabilization was within 0.1 K. The isomer shifts are given vs. metallic Fe at room temperature. The recorded spectra were fitted using a Recoil program [27].

*Nuclear inelastic scattering of synchrotron radiation.* The nuclear inelastic scattering of synchrotron radiation experiment was performed at the Nuclear Resonance beamline ID18 [28] at the European Synchrotron Radiation Facility in Grenoble, France. The storage ring was run in hybrid mode, providing 24 groups of 8 radiation pulses with the period of 88 ns. X rays were monochromatized to the energy bandwidth of 0.5 meV. The energy of incident radiation was tuned around the 14.4 keV energy of $^{57}$Fe nuclear transition within the range from -40 meV to 100 meV for 60 K and from -80 meV to 100 meV for 240 K. More details on the experimental method and setup are described elsewhere [28, 29]. A statistically meaningful spectrum of iron vibration modes in RC was obtained after 10-12 h of data collection, depending on the $^{57}$Fe concentration in the sample.
3. Results and Discussion

Typical Mössbauer spectra of the untreated and exposed to approximately a 200-fold excess of CdCl₂ chromatophors and RCs isolated from *Rs. rubrum* are presented in Fig. 3. The spectra were collected at 85 K.

In the case of samples treated with Cd²⁺ the signal is weaker because a lower amount of RCs was available due to losses during the purification procedure following the treatment. In the theoretical evaluations of the Mössbauer spectra we applied two approaches: (a) assuming quadrupole splitting distribution for the central doublet or (b) superposition of two quadrupole doublets with different quadrupole splittings and similar isomer shifts. From the distributions of the quadrupole splitting (see insets in Fig.3) we found that the central doublet detected for chromatophores and RCs can be approximated by two doublets with the line-width of 0.21 ± 0.02 mm/s and 0.19 ± 0.02 mm/s, respectively. The subspectrum with IS (isomer shift) = 0.41 ± 0.01 mm/s and QS (quadrupole splitting) = 1.02 ± 0.07 mm/s for chromatophores and IS = 0.38 ± 0.02 mm/s and QS = 1.05 ± 0.05 mm/s for RC’s is assigned to the heme iron (HFe) from the cytochromes. From gel electrophoresis we learnt that in the purified RC a contamination of cytochrome c₂ is present in addition to the RC core.

**Figure 3** The $^{57}$Fe Mössbauer spectra of chromatophores and reaction centers isolated from *Rs. rubrum*, left panel - control and right panel - treated with CdCl₂. The spectra were measured at 85 K. The lines represent fits assuming symmetrical doublets. Subspectra correspond to NHFe and HFe in cytochrome c₂. Quadrupole splitting distributions in the subsequent cases are presented in the insets.
proteins binding NHFe (data not shown). This HFe is in a low spin ferrous state [30]. The second component with IS = 0.40 ± 0.01 mm/s for chromatophores and IS = 0.37 ± 0.02 mm/s for RCs and characterized by QS = 0.58 ± 0.10 mm/s comes from NHFe (in chromatophores one expects also a contribution of some Fe-S proteins to this subspectrum). Such hyperfine parameters are characteristic for low spin Fe$^{2+}$ [16]. Finally, the spectrum obtained for the control sample of chromatophores contains a third component with IS = 0.97 ± 0.02 mm/s and QS = 2.53 ± 0.14 mm/s, typical hyperfine parameters for the high spin Fe$^{2+}$ state. This component is usually ascribed to NHFe, which high spin ferrous state in photosynthetic RC of type Q is well known [10, 18]. In order to improve the fit of the spectrum collected for control RCs one can also add a minor contribution of this high spin ferrous state but IS in this case is shifted to 1.18 ± 0.10 mm/s. The discussed above components are presented in Fig.3.

The most interesting results concern NHFe from the quinone-iron complex (QA–Fe–QB). Surprisingly, NHFe in Rs. rubrum RCs exists predominantly in a low spin ferrous state. It is believed that NHFe should exist solely in a high spin ferrous state [18, 31] in photosynthetic RCs although this diamagnetic state of NHFe has been already observed in mutated BBY PSII isolated from algae [16, 17] and a similar Mössbauer spectrum of Rs. rubrum chromatophores with only a small contribution of the high spin NHFe was detected [9]. Moreover, some EPR results can be interpreted as a signature for the presence of LS NHFe in Rs. rubrum chromatophores since otherwise it would not be possible to observe an electron paramagnetic resonance (EPR) signal having a g-value of 2.0050 ± 0.0003 characteristic for a semiquinone molecule in the whole cells of Rs. rubrum [32]. The same EPR signal of the primary quinone acceptor QA in its semiquinone form was monitored in the absence of this iron atom [33, 34, 35].

Here, we also show that Cd$^{2+}$ at molar ratio to RC as high as 200:1 does not remove the NHFe. Nevertheless, as seen in the Mössbauer spectrum (Fig. 3), after the CdCl$_2$ treatment of chromatophores (RC) NHFe exists only in the low spin state. Only two components were found in these samples, described by the following hyperfine parameters, (i) for NHFe in chromatophores: IS = 0.34 ± 0.02 mm/s and QS = 0.54 ± 0.05 mm/s, and in RCs: IS = 0.32 ± 0.01 mm/s, and QS = 0.55 ± 0.07 mm/s (ii) for HFe from cytochrome in chromatophores: IS = 0.35 ± 0.01 mm/s and QS = 0.97 ± 0.04 mm/s, and in RCs: IS = 0.35 ± 0.02 mm/s, and QS = 1.08 ± 0.05 mm/s.

For chromatophores we could observe that cadmium ions caused the transfer of NHFe high spin state into a low one. In the case of the low spin ferrous state of NHFe, the tendency of the isomer shift decrease can be noticed in the presence of the cadmium cations. These effects suggest that Cd$^{2+}$ is directly bound in the vicinity of the iron-quinone complex affecting the arrangement of the iron – ligands. No direct interaction of Cd$^{2+}$ ions with HFe of the cytochrome is observed.

![Figure 4](image-url) Figure 4 The energy dependencies of nuclear inelastic absorption in RCs isolated from Rs. rubrum enriched in $^{57}$Fe: native (A) and incubated with CdCl$_2$ (B) measured at 60 K and at 240 K. The spectra are normalized so that their first energy moments are equal to the recoil energy of a free iron nucleus. In this presentation, they show the density of absorption probability.
The experimental data of nuclear inelastic scattering indicates that the presence of Cd$^{2+}$ in the vicinity of the iron-quinone complex may influence the transfer of vibrations of the surrounding protein matrix on the NHFe bonds. The measurements were performed at two temperatures, 60 K and 240 K because of expectations of some differences in fluctuations above $200 \pm 20$ K (the characteristic temperature above which the electron transfer between $Q_A$ and $Q_B$ within the iron-quinone complex is activated). Fig. 4 shows that the spectra of the control and incubated with CdCl$_2$ RCs, measured at 60 K, differ from those collected at 240 K. As one expects at the higher temperature a significant enhancement of the low energy vibrations is observed which is caused by the increase of the population of these vibrational states with temperature. However, the comparison between the recorded spectra of the control and incubated with CdCl$_2$ RCs measured at the same temperature are much more interesting. The differences in the inelastic fluctuations are more visible in the density of vibrational states (DOS) calculated from the spectra after subtraction of the elastic contribution using the instrumental function measured in parallel [28]. The comparison of DOS obtained for control RCs and those incubated with CdCl$_2$ is presented in Fig. 5.

![Figure 5](image-url)

**Figure 5** The iron-partial density of vibrational states, DOS, of the native and treated with CdCl$_2$ RCs isolated from *Rs. rubrum* measured at 60 K (A) and at 240 K (B). The reduced DOS of the native and treated with CdCl$_2$ RCs measured at 60 K (C) and at 240 K (D). The error bars along the horizontal line (A and B) evaluate the statistical reliability of the data. The insets show the mentioned in the text differences in the DOS spectra.

The low frequency vibrations originate from the resonant $^{57}$Fe bound in the quinone-Fe complex due to the efficient transfer of protein fluctuations to NHFe bonds because only this atom is directly bound to the protein matrix. Thus, NHFe is more strongly connected to a big fragment of the protein core of RC than it is observed in the case of heme-proteins. It participates in the fast collective motions of the surrounding protein matrix fragment. The increase of the coupled mass of the protein matrix to NHFe (the increase of the moment of inertia) results in the decrease of the energy of the vibrational states [36, 37]. Therefore, in RCs from *Rs. rubrum*, NHFe vibrations dominate at low energies similarly to Fe-S clusters which have several bonds to the protein matrix [38]. It was shown in [39, 40] that the heme-Fe vibrations, regardless of whether heme is bound to protein or not, contribute significantly to the DOS at energies higher than 30 meV (in cytochrome c even higher than 35 meV) and they show only a very low contribution in the range of low vibrational frequencies (< 30 meV). Thus, the differences observed for energies < 30 meV (discussed below) come from the NHFe binding site modified by Cd$^{2+}$. At 60 K Cd$^{2+}$ cations cause damping of fluctuations within the range of 23 - 27.5 meV (Fig.5A and C), which for our statistics is slightly larger than the statistical error. At 240 K the main statistically significant differences in DOS between the control and treated with CdCl$_2$...
RCs occur at energies from 3.5 to 16.5 meV. Within this range of energies the contributions of the fluctuations detected for the control RCs are damped by Cd²⁺. This effect is much more pronounced in Fig. 5D which shows the densities of vibrational states divided by the square of the energy, i.e., the so-called reduced DOS. Such presentation enhances differences in the vibrational states between the control and CdCl₂ treated RCs within the low energetic spectrum, observed at 240 K. The data shows that NHFe in RCs in the presence of Cd²⁺ has smaller contribution in the low-energy vibrational states and, consequently, feels less massive fragment of the RC protein core. This may result from the increased force constants of bonds between NHFe and its ligands from its first coordination sphere in comparison to the force constants of bonds from the more distant protein matrix. This decreasing contribution of vibrations at low energies due to the action of Cd²⁺ ions is especially interesting because it is important for understanding of the molecular mechanism related to the down regulation of ET between the two external quinone acceptors QA and QB by cadmium cations. It was observed that Cd²⁺ ions may slow down the electron and proton transfer within the iron-quinone complex [19, 35].

4. Conclusions

The presented results shed new light on the role of NHFe in the Q-type RCs. The Mössbauer experiments demonstrate that NHFe in *Rs. rubrum* is mainly in the low spin ferrous state while usually it occurs in a ferrous high spin state. Exposure of RCs to Cd²⁺ ions causes transfer of either the high or low spin state into a new low spin state. This indicates that Cd²⁺ binds in the vicinity of the iron-quinone complex and it does not remove NHFe from this complex (at least at the applied concentrations).

The NIS experiments provide a first direct evidence on the regulatory role of the NHFe on the activation of ET within the QA – Fe – QB complex. These studies performed on the RCs treated with CdCl₂ confirmed that Cd²⁺ ions are bound in the vicinity of the quinone-Fe complex and that they can influence the low energy protein fluctuations causing their damping, especially at 240 K. Because ET between these two quinones is down regulated by Cd²⁺ and is initiated only at temperatures above 200 K, we suggest that the collective fluctuations damped by cadmium cations at about 3-16 meV are responsible for a proper activation of the coupling between QA and QB and efficient ET. This means that a certain flexibility of the RC core is required for the efficient action of the Q-type photosystem. This process can additionally be accompanied/regulated by protonation and deprotonation of residues located near NHFe as well as near the QA and QB ubiquinones binding sites (see Fig. 1B). This, in turn, may not only affect the strength of coordination bonds to NHFe but also regulate its redox potential.

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