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Short Communication

EPR evidence for a fast-relaxing iron center in Na^+-translocating NADH:quinone-oxidoreductase

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ABSTRACT

A paramagnetic Cys_4[Fe] center was detected by pulse EPR in Na^+-translocating NADH:quinone-oxidoreductase (Na^+-NQR) by influence of this center on transverse and longitudinal spin relaxation of Na^+-NQR flavin radicals. The oxidation state of the Cys_4[Fe] center was Fe^{3+} in the oxidized and Fe^{2+} in the reduced Na^+-NQR, as deduced from the temperature dependence of spin relaxation rates of different flavin radicals. A high-spin state of iron in the Cys_4[Fe] center was assigned to both forms of Na^+-NQR.

Na^+-translocating NADH:quinone-oxidoreductase (Na^+-NQR) is a component of the bacterial respiratory chain that generates transmembrane electrochemical sodium potential [1]. The enzyme consists of six subunits (NqrA-F) [2] and contains two covalently bound FMN molecules in subunits NqrB and NqrC, a [2Fe-2S] cluster, as well as noncovalently bound FAD and riboflavin (Rf) as cofactors [3]. The recently determined 3D structure of Na^+-NQR has revealed an additional prosthetic group: Fe ion coordinated by four cysteine residues between subunits NqrD and NqrE (the Cys_4[Fe] center) [4]. The Cys_4[Fe] center apparently mediates electron transfer from the [2F-2S] cluster to FMN_C, which localize at different sides of the membrane and are therefore too far apart for a direct electron transfer between them [4]. Earlier experiments failed to detect Cys_4[Fe] in Na^+-NQR using CW EPR spectroscopy [5], and the presence of this center in the enzyme was even questioned [6]. To solve this conundrum, we reinvestigate spectral properties of Na^+-NQR using CW and pulse EPR.[1]

Two Na^+-NQR forms[2] were used for EPR experiments: "as isolated" (Na^+-NQR^{ox}, oxidized form) and dithionite-treated (Na^+-NQR^{red}, reduced form). Each of these Na^+-NQR forms is known to contain a stable flavosemiquinone in about 1:1 stoichiometry to Na^+-NQR [7]. Site-directed mutagenesis, electron nuclear double resonance spectroscopy and redox titration data allowed to assign flavosemiquinone in the oxidized enzyme to a neutral radical of riboflavin (RfH·) and in the reduced enzyme to an anionic radical of FMN covalently bound to subunit NqrB ($FMN_B{\cdot}^-$) [7–11]. The other Na^+-NQR-bound FMN molecule (FMN_C), with a midpoint potential for semiquinone/hydroquinone conversion of −275 mV [10,11], is in a completely reduced form in the presence of dithionite [9]. Accordingly, the echo-detected EPR spectrum of Na^+-NQR^{ox} exhibited a single line attributable to RfH· (Fig. 1, inset). The Na^+-NQR^{red} spectrum consisted of a narrow line centered at $g \approx 2.0035$ from $FMN_B{\cdot}^-$ and a broad line with a maximum at $g = 1.94$ from the reduced [2Fe-2S] cluster [7–11].

Rubredoxin-like Cys_4[Fe] centers usually exhibit a characteristic EPR spectrum in the oxidized state, with the main rhombic signal at $g = 4.3$ [12]. However, no spectra attributable to Cys_4[Fe] were detected for both Na^+-NQR forms either in continuous wave (CW) or in pulse EPR modes, consistent with earlier findings [5]. The concentration of rhombic Fe determined from the intensity of the signal at

Abbreviations: CW, Continuous wave; Cys_4[Fe], A Fe ion coordinated by four cysteine residues; ESE, Electron spin echo; $FMN_B{\cdot}^-$, Anionic radical of FMN covalently bound to subunit NqrB; GO, Glucose oxidase; Na^+-NQR, Na^+-translocating NADH:quinone-oxidoreductase; Na^+-NQR^{ox} and Na^+-NQR^{red}, Oxidized and reduced forms of Na^+-NQR, respectively; Rf, Riboflavin; RfH·, Neutral radical of riboflavin; ZFS, Zero-field splitting

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[1] EPR experiments were performed using an X-band Elexsys-580 FT EPR spectrometer equipped with a dielectric cavity (Bruker ER 4118 X-MD-5) inside an Oxford Instruments CF 935 cryostat, which was cooled by helium flow. Na^+-NQR concentration was ~50 μM.

[2] Na^+-NQR was isolated from *Vibrio harveyi* as described previously [10]. To eliminate Fe ions nonspecifically bound to Na^+-NQR, the enzyme preparation was additionally diluted with a medium containing 100 mM KCl, 10 mM Tris-HCl (pH 8.0), 0.05% *n*-dodecyl β-D-maltoside and 1 mM EDTA (2 mM Na^+) and concentrated using a 100 kDa cut-off centrifugal filter. Of note, this EDTA treatment did not affect NADH dehydrohenase and quinone reductase activities of Na^+-NQR measured in the presence of 25 mM Na^+. When required, the enzyme was converted to its reduced form by adding 20 mM disodium dithionite.

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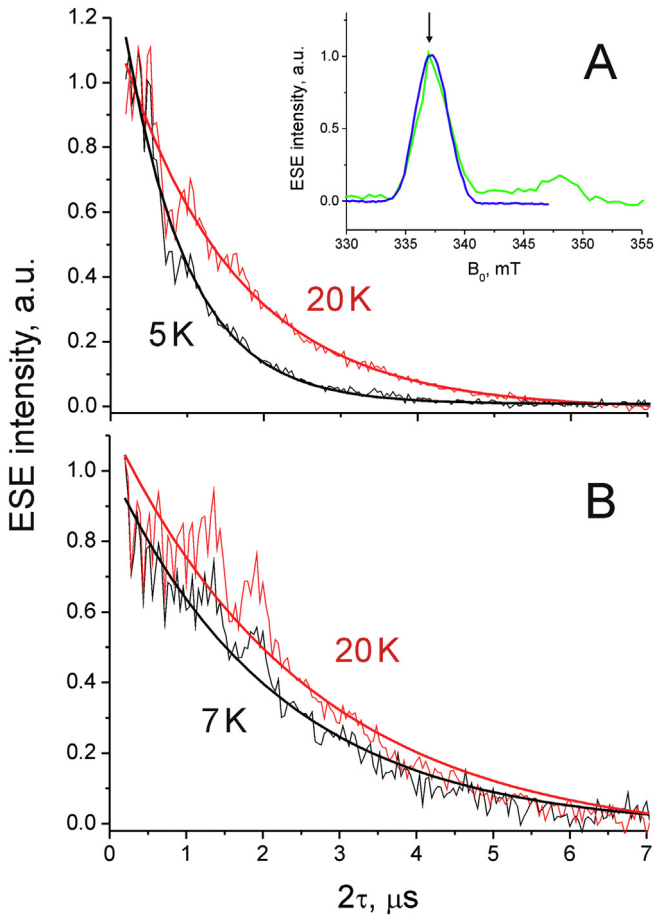

Fig. 1. Panel A: Two-pulse ESE decay at 5 K (black thin curve) and 20 K (red thin curve) and their monoexponential fits (thick curves) for flavin radical in Na^+-NQR^{ox}. Panel B: The same for Na^+-NQR^{red}, temperature 7 K (black curves) and 20 K (red curves). Inset: echo-detected EPR spectra for Na^+-NQR^{red} (green curve) and Na^+-NQR^{ox} (blue curve) at 5 K. Duration of $\pi/2$ and π microwave pulses 12 and 24 ns, respectively, interpulse delay τ = 120 ns, shot repetition time 2 s. The arrow marks the spectral position where spin relaxation experiments were performed (g = 2.0035). Weak broad EPR line with maximum at 348 mT belongs to [2Fe-2S] cluster in Na^+-NQR^{red}. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

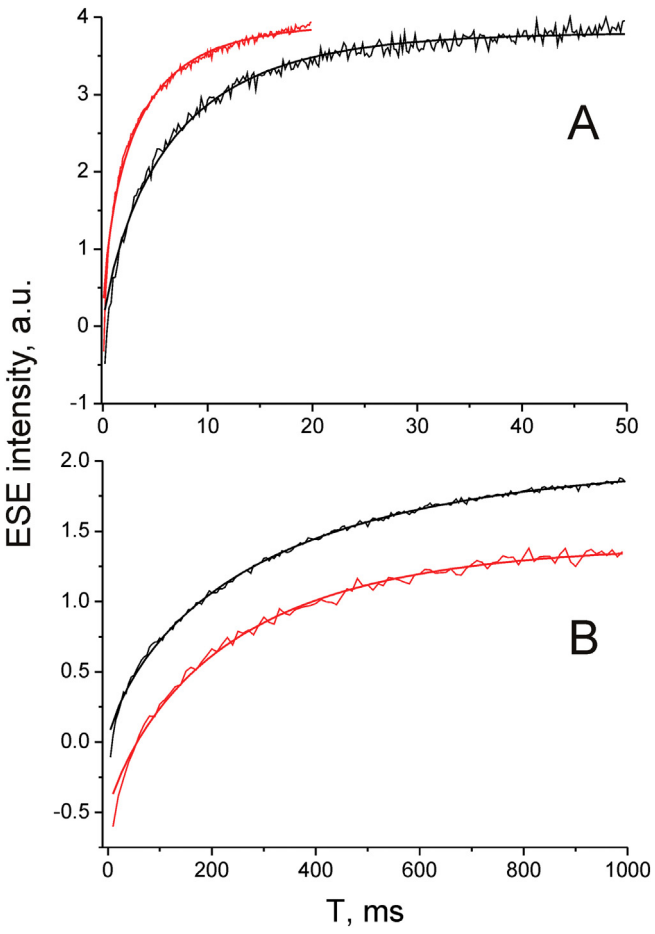

Fig. 2. Panel A: Inversion-recovery traces obtained by microwave pulse sequence π–T–$\pi/2$–τ–π–τ–echo for flavin radicals in Na^+-NQR^{ox} (red thin curve) and Na^+-NQR^{red} (black thin curve). All these curves can be fitted to a stretched exponential, which is typical for longitudinal relaxation enhancement [24], from which the effective T_1 value can be determined. Their approximation of the inversion-recovery traces by stretched exponential is shown by thick curves. Panel B: Inversion-recovery traces for anionic (black thin curve) and neutral (red thin curve) flavin radicals in GO and their approximation by stretched exponential (thick curves). Since the inversion-recovery traces are nearly identical for the anionic and neutral flavin radical in GO, the latter is shifted downwards. The temperature is 20 K for all traces. Note the different timescales for panels A and B. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

g = 4.3 (Fig. S1) [13] was < 1 μM for Na^+-NQR^{red} and ≤ 3 μM for Na^+-NQR^{ox}, i.e. much smaller than the Na^+-NQR concentration estimated from the radical CW EPR signal intensity, 35 μM.[3] Therefore, the weak g = 4.3 EPR signal in Na^+-NQR apparently originates from non-specifically bound Fe. The same results were obtained at lower temperatures (down to 4 K).

The inability to detect Cys_4[Fe] by EPR can be explained by its very short spin relaxation times. Therefore, we attempted to detect Cys_4[Fe] indirectly by transverse (T_2) and longitudinal (T_1) spin relaxation enhancement of flavin radicals. In this approach, slow-relaxing spins of radicals are used to probe magnetic interaction with fast-relaxing paramagnetic ions [14]. For RfH· in Na^+-NQR^{ox}, the maximal transverse relaxation rate $1/T_2$ was observed at very low temperature (Fig. 1A), which is typical for spins of organic radicals coupled with a fast-relaxing Fe ion [15]. For Na^+-NQR^{red}, a similar but much smaller effect on $1/T_2$ was observed, with a maximum achieved at a higher temperature (Fig. 1B). Relaxation enhancement of radicals in Na^+-NQR cannot be caused by interaction with the [2Fe-2S] cluster because it is in a diamagnetic state in Na^+-NQR^{ox} and its longitudinal relaxation in Na^+-NQR^{red} is too slow (T_1 > 100 μs at temperatures below 20 K, see Fig. S2). As Na^+-NQR does not contain metals other than Fe [5], we assign the relaxation enhancement of Na^+-NQR radicals to their interaction with Cys_4[Fe].

The effect of Cys_4[Fe] on longitudinal relaxation of radicals in Na^+-NQR was more pronounced (Fig. 2A). The observed relaxation rate was noticeably faster for Na^+-NQR^{ox}. Analogous magnetization recovery curves for the anionic and neutral flavin radicals in glucose oxidase (GO),[4] which do not experience relaxation enhancement by paramagnetic ions, were nearly identical (Fig. 2B) and demonstrated much slower relaxation than the Na^+-NQR radicals.

Fig. 3 compares spin relaxation rates for Na^+-NQR and GO radicals. First, a dramatic increase of $1/T_2$ was observed at low temperature for

[3] The concentration of flavin radicals in both Na^+-NQR samples, 35 μM, was estimated from double integrals of CW EPR spectra taken at 100 K and compared with the analogous integral of the CW EPR spectrum of a $CuCl_2{\cdot}2H_2O$ crystal of known mass.

[4] To produce neutral and anionic flavin radicals in *Aspergillus niger* glucose oxidase, anaerobic samples of the enzyme were photoreduced at pH 5.8 and 10.2, respectively, as described elsewhere [7,22].