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Resonance Raman spectra of the FMN of the bovine heart NADH: ubiquinone oxidoreductase, the largest membrane protein in the mitochondrial respiratory system

Hidehisa Sugiyama · Ryohei Nakatsubo ·
Satoru Yamaguchi · Takashi Ogura ·
Kyoko Shinzawa-Itoh · Shinya Yoshikawa

Abstract The resonance Raman spectra of FMN of the bovine heart NADH: ubiquinone oxidoreductase with the molecular mass of approximately one million dalton were determined by using a highly improved enzyme preparation and a resonance Raman apparatus. The band positions and the H_2O/D_2O exchange effect suggest that the $N(3)-H$ group in the ring III of the isoalloxazine moiety is buried inside the protein to increase the vibrational coupling to the $C(2)-N(3)-C(4)$ stretching mode and that the ring I is exposed to the aqueous phase.

Keywords NADH: ubiquinone oxidoreductase · FMN · Respiratory chain · Resonance Raman

Abbreviations FMN: (Flavin mononucleotide) · PHBH: (*para*-hydroxybenzoate hydroxylase)

Introduction

NADH: ubiquinone oxidoreductase (EC1.6.5.3) (complex I) reduces ubiquinone by NADH at the entrance of the mitochondrial electron transfer chain. The electron transfer reaction is coupled to proton pumping with a stoichiometry of $4H^+/2e^-$ (Hatefi et al., 1961). The enzyme is the largest component of the mitochondrial respiratory system with the proposed mass of approximately 1000 kDa composed of 45 subunit types (Walker, 1992; Hirst et al., 2003). The putative

H. Sugiyama · R. Nakatsubo · S. Yamaguchi · T. Ogura (✉) ·
K. Shinzawa-Itoh · S. Yoshikawa
Department of Life Science, Graduate School of Life Science,
University of Hyogo, Koto 3-2-1, Kamigori-cho, Ako-gun, Hyogo
678-1297, Japan
e-mail: ogura@sci.u-hyogo.ac.jp

redox-active sites include six EPR detectable iron-sulfur clusters, one FMN and several tightly bound coenzymes Q. Steady state kinetic analyses indicate that the NADH binding is tightly coupled to the ubiquinone binding, suggesting various long-range interactions between these redox sites.

In order to elucidate the reaction mechanism of complex I, spectral characterizations of these redox sites are prerequisite as well as X-ray structural analyses at high resolution. Although extensive EPR investigations for the iron-sulfur clusters have identified the redox and magneto chemical properties of the clusters (Ohnishi et al., 1985; Ohnishi, 1998), no spectral and functional study has been reported for the flavin moiety, since sufficient removal of contaminant proteins with strong visible absorptions was impossible. The absorption spectrum of the isolated enzyme was reported for the first time as late as in 2002, about 40 years later since the discovery of the enzyme (Nakashima et al., 2002). The enzyme preparation contained a negligible amount of hemoproteins (less than 2% of cytochrome *c* oxidase (mole/mole)). The preparation of complex I prepared by ourselves following the method of Hatefi (Hatefi et al., 1961; Hatefi, 1978) contained at least 15% of the contaminant cytochrome *c* oxidase. Furthermore, the absorbance of the iron-sulfur clusters would mask significantly the flavin spectrum even if contaminant proteins are completely removed. Thus, no absorption spectral analysis for the flavin site has been reported.

Resonance Raman technique is powerful for identification of a chromophore contained in a multichomophore system. However, contaminant proteins with much stronger absorptions than that of FMN, like cytochrome *c* oxidase, seriously obstruct accurate measurement of the resonance Raman spectrum of the FMN moiety of complex I. Furthermore, in general, the signal-to-noise ratio of the Raman spectrum is lower for a larger protein, because Rayleigh scattering intensity is proportional to the mass of a molecule. Rayleigh scat-

tering, which is much stronger than Raman scattering, causes stray light in a spectrograph of a Raman apparatus to result in high background to the measurement. In this case, raising the sample concentration cannot be a help to record resonance Raman spectra with higher quality. The sensitivity and accuracy of a Raman spectrophotometer greatly depends on its efficiency for lowering the level of the stray light in the apparatus. In fact, the sizes of flavoproteins, the resonance Raman spectra of which have been measured thus far, are 65 kDa or smaller (Kitagawa et al., 1979; Altose et al., 2001). No attempt has been made for the resonance Raman measurement of FMN of complex I with the molecular mass of 1000 kDa. The highly improved enzyme preparation and Raman apparatus in the present study were critical for obtaining the resonance Raman spectrum of the FMN of complex I at this accuracy.

Materials and methods

Complex I was purified from bovine heart muscle as reported (Nakashima et al., 2002). The integrity of the purified enzyme was monitored by the absorption spectrum, the turnover enzyme activity (NADH oxidation in the presence of decylubiquinone) and the sensitivity to an enzyme inhibitor, piericidin. The enzyme preparation used for the present investigation contained one FMN/1000 kDa of the protein.

Resonance Raman scattering was excited at 441.6 nm from a He-Cd laser (Kimmon Koha Co., Ltd., IK5651R-G) and detected with an intensified photodiode array (1024 pixels, PAR 1421HQ) attached to a single spectrograph (Ritsu Oyo Kogaku Co., Ltd., MC-100DG). A holographic notch filter (Kaiser Optical Systems, Inc., HNPF-442.0) was used to reduce the interference of Rayleigh scattering to Raman spectra. A quartz spinning-cell (inner diameter = 5 mm, 1200 rpm) was used to avoid local heating and degradation of the sample. A small magnet was employed to effectively stir the sample solution as described elsewhere (Aki et al., 2000). Flushing low temperature nitrogen gas against the cell to keep the temperature at $8°C$ was essential to maintain the integrity of the sample during Raman measurements. In order to compensate the sensitivity difference of each pixel of the diode array, each Raman spectrum was divided by the "white light" spectrum, which was obtained by measuring the scattered radiation of an incandescent lamp by a white paper. A linear base line was subtracted from the resultant spectrum to remove fluorescence background. Raman frequencies were calibrated with indene as a frequency standard.

For efficient reduction of the unnecessary light, accurate alignment of the incident angle of the holographic notch filter is critical, since the rejecting wavelength is sensitive to the

incident angle. For this purpose, the notch filter was mounted on an angle-adjustable mirror holder for fine-tuning of the incident angle. The device was essential for improvement of the sensitivity and accuracy of the apparatus sufficiently high for obtaining the resonance Raman spectra of FMN in complex I.

Results and discussion

Figure 1 shows the resonance Raman spectra of complex I as prepared in D_2O (A), H_2O (B) and the difference spectrum (C). Because of the high back-ground due to the large-sized protein, spectrum accumulation for 96 min was required for obtaining the signal to noise ratio as given in the figure. The band at 980 cm^{-1} is due to the sulfate ion (SO_4^{2-}) of residual ammonium sulfate in the isolated preparation. All the Raman bands above 1200 cm^{-1} are those assignable to the modes of the oxidized isoalloxazine ring. These band positions are essentially identical with those of the riboflavin bound to the egg yolk riboflavin binding protein. Only the 1251 cm^{-1} band shows an up-shift to 1288 cm^{-1} upon H_2O to D_2O exchange. The 1251 cm^{-1} band in H_2O has been assigned to $C(2)-N(3)-C(4)$ stretching vibration coupled with $N(3)-H$ bending vibration in the ring III of the isoalloxazine moiety (Kitagawa et al., 1979). Upon H/D exchange the coupling is broken to show an up-shift of the 1251 cm^{-1} band to 1288 cm^{-1}. No other band located in the higher wave number region than 1226 cm^{-1} is influenced by the exchange. The H_2O/D_2O exchange effect is similar to that of the riboflavin in the egg yolk riboflavin binding protein. The band position in H_2O (1251 cm^{-1}) is near that of the out-conformation of para-hydroxybenzoate hydroxylase (PHBH) (1255 cm^{-1}) and significantly higher than that of the in-conformation (1238 cm^{-1}) (Altose et al., 2001). X-ray structural analyses of PHBH indicate that the isoalloxazine moiety is essentially solvent-exposed and buried in the protein in the out- and in-conformations, respectively. The band position of complex I is closer to the out-conformation. However, the X-ray structure of FMN of complex I recently determined indicates that the ring III side of the isoalloxazine moiety of complex I is essentially buried in the protein matrix, exposing only the $C(7)-C(8)$ edge of the ring I (Sazanov and Hinchliffe, 2006). The overall conformation is similar to the in-conformation of PHBH. At the present resolution of X-ray structure of complex I, it is difficult to identify the structure of protein moiety of complex I which provide the weaker vibrational coupling compared with that of PHMB. These results indicate that resonance Raman spectroscopy and X-ray structural analyses are compensative for analyses of the protein-flavin interactions. The 1407 cm^{-1} band suggests that ring I of the isoalloxazine ring is exposed to the aqueous phase consistent with the X-ray structural results.
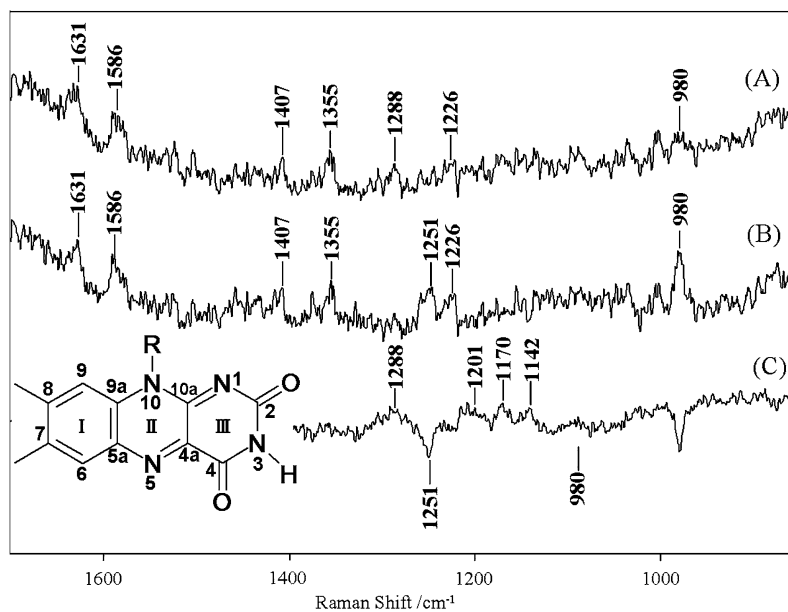
Fig. 1 Resonance Raman spectra of the oxidized complex I in D_2O (A), H_2O (B) and the difference, $D_2O–H_2O$ (C). The enzyme concentration in terms of FMN is 80 μM in 40 mM HEPES buffer pH 7.8 containing 600 mM Sucrose. Each resonance Raman spectrum was measured upon excitation at 441.6 nm. The upper two spectra were obtained by accumulation of 1800 measurements of 3.2 sec exposure (5760 sec). The ordinate scales of the three spectra are identical. The lower noise level in the difference spectrum (spectrum C) suggests that differences in the character of the sensitivity between each pixel of the diode array are not negligible even after compensation by white light. The Raman bands at 1201, 1170 and 1142 cm^{-1} of the D_2O sample were thus uncovered after subtraction (Spectrum C)

All these Raman bands except for the one at 980 cm^{-1} disappeared by addition of excess amount of NADH (25 mM). Excess amount of dithionite also eliminated these bands due to the FMN. The Raman band assignable to the fully reduced cytochrome c oxidase appeared in the presence of dithionite, since the excitation wavelength at 441.6 nm is located close to the absorption peak of Soret band of the reduced cytochrome c oxidase. When NADH is used as the reductant, Raman bands due to NADH appeared at 1687, 1112 and 1078 cm^{-1} without giving the bands due to the fully reduced cytochrome c oxidase, since NADH does not reduce cytochrome c oxidase (Data not shown). In spite of the presence of various bands due to the fully reduced cytochrome c oxidase and NADH in the wave number region of the oxidized FMN bands, the absence of any FMN band was obvious in both the spectra for complex I reduced with either dithionite or NADH, since these Raman bands are sharp enough to resolve with each other. No significant bands due to cytochrome c oxidase is detectable in the oxidized state as shown in Fig. 1, since the Raman scattering intensities of the contaminant oxidized cytochrome c oxidase in the improved preparation are not strong enough to give detectable resonance Raman spectra.

The solvent accessibility revealed by H_2O/D_2O exchange is the first experimental support for the function of the FMN moiety as the initial hydrogen acceptor from NADH. Thus, the FMN moiety is most likely to be the NADH-binding site to receive electrons directly as stated above. The present results provide basic information for elucidation of the mechanism of the long-range interactions between NADH and ubiquinone binding sites and between FMN and other redox active sites as suggested by the steady state kinetic analyses (Nakashima et al., 2002). To our knowledge, complex I is the biggest flavoprotein, the resonance Raman spectrum of which has been obtained thus far. No other method than this improved resonance Raman system would be applicable to examine the spectral property of the FMN in complex I. Further improvement of the purification method is under way for the stability and purity of the enzyme preparation which is critical for higher signal to noise ratio of the resonance Raman measurement.

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