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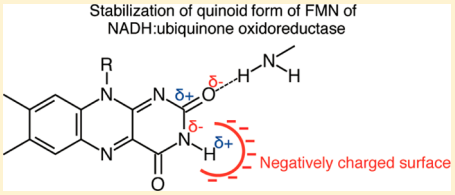
Resonance Raman Spectral Properties of FMN of Bovine Heart NADH:ubiquinone Oxidoreductase Suggesting a Mechanism for the Prevention of Spontaneous Production of Reactive Oxygen Species

Masahide Hikita, Kyoko Shinzawa-Itoh, Masakazu Moriyama, Takashi Ogura, Kiyohito Kihira, and Shinya 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

ABSTRACT: A highly improved method for obtaining resonance Raman (RR) spectra provided spectra comparable to the best known flavoprotein spectra when the method was tested using bovine heart NADH:ubiquinone oxidoreductase (Complex I), a protein with a molecular mass of 1000 kDa, which causes the level of RR noise to be 1 order of magnitude higher than for most flavoproteins. The FMN RR band shift (1631/1633 cm^{-1}) and the increase in the magnitude of the band at 1252 cm^{-1} upon binding to Complex I suggest hydrogen bond formation involving one of the C═O groups [C(2)═O] of isoalloxazine to stabilize its quinoid form. This lowers the redox potential of FMN and the electron density of the O_2 binding site [a carbon atom, C(4a)] in the reduced form. Thus, spontaneous production of reactive oxygen species at the FMN site is prevented by minimizing the duration of the fully reduced state by accelerating the FMN oxidation and by weakening the O_2 affinity of C(4a). Other band shifts (1258/1252 cm^{-1} and 1161/1158 cm^{-1}) suggest a significantly weaker hydrogen bond to the NH group [N(3)-H] of isoalloxazine. This result, together with the reported X-ray structure in which N(3)-H is surrounded by negatively charged surface without hydrogen bond formation, suggests that N(3)-H is weakly but significantly polarized. The polarized N(3)-H, adjacent to the C(2)═O group, stabilizes the polarized state of C(2)═O to strengthen the hydrogen bond to C(2)═O. This could fine-tune the hydrogen bond strength. Other results show a high-dielectric constant environment and weak hydrogen bonds to the isoalloxazine, suggesting adaptability for various functional controls.



NADH:ubiquinone oxidoreductase (EC 1.6.5.3, Complex I) promotes NADH-dependent reduction of ubiquinone at the entrance of the mitochondrial electron transfer chain. Electron transfer through Complex I is coupled to the proton pumping process with a stoichiometry of four protons per two electrons.[1] The enzyme is the largest component of the mitochondrial respiratory system and has a proposed mass of approximately 1000 kDa with 45 different subunits.[2] The composition of the cofactors (or nonprotein components) of bovine heart Complex I has been determined recently[3] and includes one ubiquinone with 10 isoprenoid units, 70 phosphorus atoms of phospholipids, one zinc ion, one FMN, 30 inorganic sulfur atoms, and 30 iron atoms. On the other hand, X-ray structures of the bacterial[4−6] and yeast[7] enzymes have been reported, and a proton pumping mechanism has been proposed. The X-ray structure of the flavin site has been determined at 3.1 Å resolution.[8]

For elucidation of the role of the flavin site in the catalytic cycle, extensive analyses of the resonance Raman (RR) properties of the bound FMN are needed in addition to high-resolution X-ray structures. X-ray structural analysis at high resolution would identify all of the functional groups that could interact with the bound FMN. However, for example, the strength of a hydrogen bond to the FMN cannot be evaluated quantitatively without Raman and/or infrared analyses, because the hydrogen bond strength is greatly influenced by subtle changes in the microenvironment of the protein without resulting in significant structural changes in the X-ray structure.

The isoalloxazine moiety of flavin can exist in three different oxidation states, the fully oxidized (quinoid), the semiquinoid, and fully reduced (leuco) states as given in Figure 1. The fully reduced form has no absorption in the visible region. Thus, no resonance enhancement of Raman bands using a visible light laser is possible for the fully reduced flavin. On the other hand, this property provides a powerful tool for the identification for flavin RR bands in other oxidation states in flavoproteins containing additional chromophores. The fully oxidized form of flavin shows 13 major bands in the higher-wavenumber region (1700−1100 cm^{-1}).[9] Each RR band includes various vibrational modes[10,11] because isoalloxazine in the fully oxidized state forms a completely conjugated π-electron system, as shown in Figure 1. Therefore, structural information provided by RR results is usually not straightforward. However, various experimental efforts aimed at band assignments, that is, effects of various structural factors on the flavin RR spectra, including isotopes, chemical modifications, solvents, and protein structures, have provided various useful spectral properties

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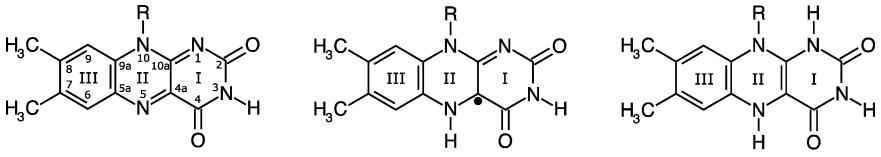

Figure 1. Isoalloxazine structures in the fully oxidized, semiquinoid, and fully reduced states. The structure in the fully oxidized state shows the numbering system.

from which structural information for the flavins sites in proteins can be obtained empirically.[10,11]

In 2007, RR spectra of bovine heart Complex I were published in an initial report on a vibrational analysis of Complex I.[12] Although the report includes interesting findings such as the D_2O-H_2O exchange effect on the 1251 cm^{-1} band that includes a vibrational mode of the NH group of the isoalloxazine, only 6 of 13 possible bands were identified between 1700 and 1100 cm^{-1} because of the low signal:noise ratio. In general, flavin-based Raman band shifts induced by changes in the microenvironment, including hydrogen bond structures, are fairly small. Thus, the accuracy of the identification of the bands, at least, as high as ± 0.5 cm^{-1} is necessary for detailed examination of the function of protein-bound flavins. The most significant problem encountered in RR spectral measurements of Complex I is the noise level induced by Rayleigh scattering that is proportional to the molecular mass and causes stray light within a Raman apparatus. This causes a high level of background noise. Various improvements in the instrumentation for RR measurements in addition to those previously reported[12] and methods for the Raman measurement and enzyme sample purification have been applied to provide desirable accuracy for RR measurements of Complex I. The RR spectra of Complex I excited with visible laser beams include bands originating from the FMN site and iron−sulfur centers. Thus, the effects of complete reduction of Complex I with its natural electron donor (NADH) on the RR spectra of oxidized Complex I are necessary to identify the RR bands due to FMN, as described above.

The RR spectra of FMN in Complex I in the oxidized state with a band determination accuracy of ± 0.4 cm^{-1}, determined here, suggest the existence of a mechanism for the prevention of spontaneous production of reactive oxygen species.

MATERIALS AND METHODS

Complex I was purified from bovine heart muscle with an improved method as previously reported.[3] Other chemicals and proteins used for this study are of the highest grade commercially available. For RR measurements, a holographic edge filter (Semrock, LP02-442RU) was used to reduce the interference of Rayleigh scattering to Raman spectra. Resonance Raman scattering was excited at 441.6 nm from a He−Cd laser (Kimmon Koha, IK5651R-G), dispersed by a single polychromator (Chromex, 500IS), and detected by a liquid N_2-cooled CCD detector (Roper Scientific, Spec-10:400B/LN). Nitrogen gas cooled by liquid nitrogen was flushed against the spinning cell to keep the temperature in the sample cell between −2 and 0 °C. To minimize the exposure of the solution of the fully reduced enzyme in the cell to atmospheric oxygen, the spinning cell was sealed with a silicone rubber septum and the gas phase above the enzyme solution was completely filled with paraffin oil washed several times by being bubbled with Ar gas. With this improvement, no significant spectral change showing oxidation of the enzyme system inside the cell under the spinning conditions required for RR measurements was detectable at least for 6 h. Other experimental conditions were described previously.[12]

RESULTS AND DISCUSSION

Improvements in the RR Measurement Method for Bovine Heart Complex I. Our RR measurements were performed using the same polychromator described previously.[12] However, replacement of the diode array detector with a liquid nitrogen-cooled CCD detector as described above and improvements in the experimental conditions described below have greatly increased the signal:noise ratio, which is obvious in the comparison of the spectra presented here with previous spectra (Figure 2).

The noise level of Complex I caused by the intensity of Rayleigh scattering was lowered by extending the spectrum

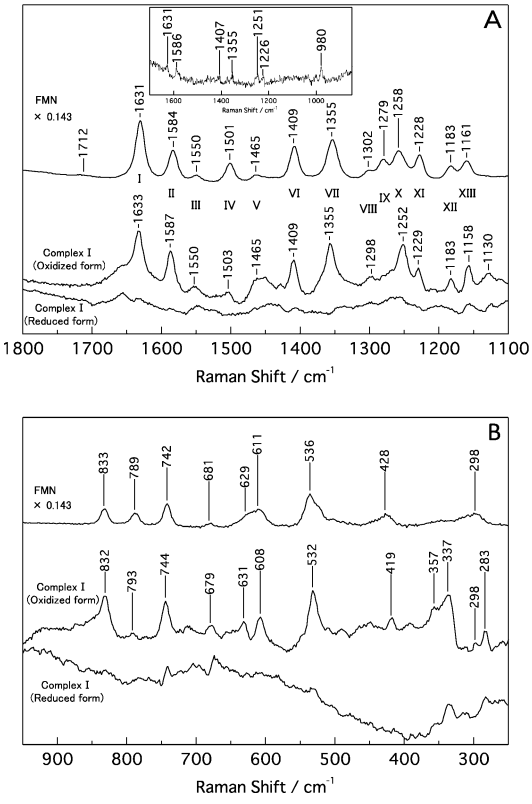

Figure 2. Resonance Raman spectra of bovine heart Complex I and FMN in aqueous solution, excited at 441.6 nm. Panels A and B provides those in the higher- and lower-wavenumber regions, respectively. The inset in panel A shows the resonance Raman spectrum published previously,[12] where the band at 980 cm^{-1} is assigned to $(NH_4)_2SO_4$. Both panels include the resonance Raman spectra of fully reduced Complex I.

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accumulation time from 96 min to 3 h at an increased laser power (3.0 mW instead of 1.5 mW). To compensate for the sensitivity difference of each pixel of the CCD, each Raman spectrum was divided by the "white light" spectrum that was determined by measuring the scattered radiation of an incandescent lamp by a white paper. The accuracy of the "white light" spectrum is critical for improvement of the signal:noise ratio of the resulting Raman spectrum. Under these experimental conditions, the optimal accumulation time for the "white light" spectrum has been found to be 9 h.

Improvements in the method of purification of the enzyme and sample temperature control during the RR measurements also critically contribute to the improvement in spectral quality. Furthermore, the edge filter used for this work has significantly improved the signal:noise ratio, especially in the spectral region between 500 and 200 cm^{-1}. This RR spectral system provides the best flavin Raman spectrum, thus far, using FMN in an aqueous solution that has a significantly high noise level even in the presence of 2 M KI (Figure 2). It should be noted that accurate RR measurements for flavins in aqueous solution, which show a high level of fluorescence, are in general much harder to acquire than those for protein-bound flavins.[10]

Effects of Complete Reduction on the RR Spectra of Complex I. To identify the FMN RR band of Complex I in the fully oxidized state, we measured RR spectra for the fully reduced Complex I sample. The Complex I sample used in this study contains <2% cytochrome *c* oxidase (in molar ratio) and trace amounts of the other electron transfer components of the mitochondrial respiratory system. Thus, sufficient anaerobiosis and a reducing system for NAD^+ are necessary to keep the Complex I sample in the completely reduced state during the RR measurement over the course of 3 h. For this anaerobiosis system that uses a spinning cell for the RR analyses as described above, a NAD^+ reducing system containing malate dehydrogenase and citrate synthase in the presence of excess acetyl CoA in a molar ratio of 2:1:180 at pH 7.8 for 40−50 μM Complex I was sufficient to keep NADH and Complex I in the completely reduced state for 6 h. The oxidation state of the enzyme system was verified by monitoring the absorbance of NADH at 340 nm.

The nonresonance Raman background due to the NADH-based reducing system was subtracted from the resulting fully reduced RR spectrum of the Complex I sample system using the ammonium sulfate band as the internal standard. The fairly intense RR bands due to fully reduced cytochrome *c* oxidase that has a Soret band (at 443 nm) very close to the wavelength of the excitation laser light (441.6 nm) were subtracted by estimation of the RR band intensity of cytochrome *c* oxidase (CcO) at each wavenumber using the most prominent cytochrome *c* oxidase band at 1355 cm^{-1} where no RR band of oxidized flavin in aqueous solution is located. Subtraction of the spectra of NADH and fully reduced CcO from the spectra of the Complex I sample in the fully reduced state decreases significantly the reproducibility of the resulting spectra compared with that of the experimentally obtained spectra before the subtraction process. In fact, the resulting spectra given in Figure 2 as a typical result show several weak peaks near the weak band positions of the fully oxidized form at 1550, 1158, 1130, 744, and 679 cm^{-1}. However, the reproducibility of the bands near 1550, 744, and 679 cm^{-1} is not sufficiently high to conclude the presence of Raman bands at these positions in the fully reduced state. The weak bands near 1158 and 1130 cm^{-1} are quite reproducibly detectable. However, the former band is definitely broader than that of fully oxidized Complex I, while the difference in the peak position of the latter band (1130 cm^{-1}) versus that of fully oxidized Complex I (1126 cm^{-1}) is obvious. Thus, these bands are unlikely to be due to the fully oxidized FMN in Complex I. The bands that can be detected near 357, 337, and 283 cm^{-1} are due to those of the iron−sulfur sites.

Band Assignments. As shown in Figure 2, most of the RR bands of fully oxidized Complex I have counterpart bands in the spectrum of FMN in an aqueous solution. These bands disappear upon complete reduction of Complex I with NADH, as shown in the spectra of reduced Complex I given in Figure 2. All of these bands can be reasonably assigned to those of FMN in Complex I. In other words, all FMN molecules in our Complex I preparation can be reduced with the natural electron donor, NADH. This indicates that the amount of denatured proteins in our preparation is negligible.

The bands at 1712 and 428 cm^{-1} that can be detected in FMN in aqueous solution seem too broad to be detected in the RR spectrum of Complex I. The intensity of the band at 1279 cm^{-1} that can be detected in FMN in aqueous solution is likely to be decreased and/or shifted to a lower-wavenumber region to overlap the 1252 cm^{-1} band of Complex I. The 1465 cm^{-1} band of methyl deformation of FMN in Complex I is located near the nonresonance Raman band of the C−H stretch of $-CH_2-$, although it is not clearly detectable in the spectrum of the fully reduced form of Complex I, because of the insufficient sensitivity of this Raman measurement. A tentative band position is shown in the figure and table. On the other hand, a counter band for the 1130 cm^{-1} band is not detectable. A reasonable assignment for this band is not possible at present.

The accuracies of determination of band positions of RR spectra of FMN in aqueous solution and of FMN in Complex I in the higher-wavenumber region between 1700 and 1100 cm^{-1} were estimated to be 0.2 and 0.4 cm^{-1} according to six and five repeated determinations, respectively. The accuracy of the RR spectral measurement in the lower-wavenumber region (900−250 cm^{-1}) is sufficiently high to detect band shifts upon binding of FMN in aqueous solution to Complex I, such as the band shifts from 789 to 793 cm^{-1}, from 742 to 744 cm^{-1}, and from 536 to 532 cm^{-1}. The accuracy of the spectral measurements in the lower-wavenumber region where the background noise level is much higher than in the higher-wavenumber region is also significantly greater than the accuracy in the lower-wavenumber region for proteins much smaller than Complex I.[13−16] These results show a revolutionary improvement in the performance of RR in measurements of protein systems.

The 419 and 283 cm^{-1} bands and the 357/337 cm^{-1} doublet band do not have counter bands in the spectrum of FMN in aqueous solution. These bands can be assigned to [2Fe-2S] and [4Fe-4S] RR bands, respectively, on the basis of the reported results for various iron−sulfur proteins.[17−20] This RR system showed that two clear RR bands, corresponding to the 419 and 283 cm^{-1} bands, are located in the RR spectrum of spinach [2Fe-2S] ferredoxin. It should be noted that clear spectroscopic data for the iron−sulfur sites of Complex I in the oxidized state have not been reported thus far (the extensive EPR measurements of the iron−sulfur centers are for the reduced form of Complex I). No band that can be assigned to the iron−sulfur sites can be detected in the higher-wavenumber region, consistent with the previous report for various iron−sulfur proteins.[17−20] Several weaker bands of the iron−sulfur sites that

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have been reported for other iron−sulfur proteins near the present iron−sulfur bands[17−20] are not detectable, probably because of the lack of sufficient sensitivity in the Raman measurement in the low-wavenumber region. Under these experimental conditions, a Raman excitation laser wavelength at 441.6 nm was selected for the RR spectrum of the flavin moiety. An alternative and improved laser system is expected to provide more accurate RR spectral measurements for the iron−sulfur sites of Complex I.

Properties of the Fully Oxidized FMN Bound to Complex I, As Shown by RR Analyses. Table 1 shows the band positions, assignments (FMN, [2Fe-2S], or [4Fe-4S]) and nomenclature of the flavin bands in the higher-wavenumber region.[9] As shown in Figure 2 and Table 1, accurate RR measurements under these conditions indicate various small but significant differences between the flavin spectra in solution and in Complex I. This has allowed us to identify critical aspects of the functions provided by the fully oxidized flavin site in Complex I.

Table 1. Raman Frequencies (cm^{-1}) of Complex I and FMN in Aqueous Solution

Complex I	FMN in aqueous solution	frequency shift ($\Delta\nu$)[a]	band number[b]
–	1712		
1633	1631	2	I
1587	1584	3	II
1550	1550	0	III
1503	1501	2	IV
1465	1465	0	V
1409	1409	0	VI
1355	1355	0	VII
1298	1302	−4	VIII
–	1279		IX
1252	1258	−6	X
1229	1228	1	XI
1183	1183	0	XII
1158	1161	−3	XIII
832	833	−1	
793	789	4	
744	742	2	
679	681	−2	
631	629	2	
608	611	−3	
532	536	−4	
–	428		
298	298	0	
419[c]	–		
357[d]	–		
337[d]	–		
283[c]	–		

[a] $\Delta\nu$ = ν(Complex I) − ν(FMN in aqueous solution). [b] Band numbering according to ref 9. [c] Can be assigned to a [2Fe-2S] center. [d] Can be assigned to a [4Fe-4S] center.

It has been proposed that bands IV (~1500 cm^{-1}), VI (~1410 cm^{-1}), and VII (~1355 cm^{-1}) are empirical markers for the dielectric environment of the isoalloxazine moiety.[21] The absence of any significant differences in these bands of Complex I versus those of FMN in aqueous solution indicates that the overall dielectric environment of FMN in Complex I is essentially identical to that of FMN in aqueous solution. The RR results are consistent with the reported X-ray structure of the FMN binding site of Complex I, which shows that various hydrophilic groups and solvent accessible spaces surround the isoalloxazine moiety.[8] This seems to be consistent with the effect of H_2O−D_2O exchange on band X, which, in turn, suggests that N(3)-H−N(3)-D exchange occurs, as reported previously[12] (the position of each atom in the isoalloxazine moiety is shown in Figure 1 and indicated in parentheses). The reported X-ray structure of the flavin site in the oxidized state indicates that solvent water molecules are accessible to the isoalloxazine moiety via the solvent accessible space on one side of the ring.[8] Thus, the reported X-ray structural analysis identifies the functional groups and structures that are likely to contribute to the overall dielectric environment of the FMN, while RR analysis experimentally assesses the overall dielectric environment produced by these surrounding groups that was identified by X-ray analysis. Thus, both X-ray and RR analyses are required for examination of the function of the FMN of Complex I. The overall dielectric environment of FMN, essentially identical to that of FMN in aqueous solution, suggests that hydrogen bonds to FMN in Complex I are significantly weaker than those buried inside the protein moiety.

The largest frequency shift can be detected in band X (~1260 cm^{-1}), which has a 6 cm^{-1} lower wavenumber in Complex I (1252 cm^{-1}), compared with the corresponding band of FMN in aqueous solution (1258 cm^{-1}). This band, together with the shift in band XIII (1161/1158 cm^{-1}), involves the N(3)-H bending mode.[10,21−23] It has been proposed that the lower-frequency shifts in bands X and XIII are induced by weakening of the hydrogen bonding environment of the N(3)-H group.[10,13,14] Thus, these lower-frequency shifts strongly suggest that hydrogen bonding to N(3)-H in Complex I is significantly weaker than that in FMN in aqueous solution. However, the band shifts induced by a large conformational change in the isoalloxazine of *p*-hydroxybenzoate hydroxylase (PHBH) from the conformation in which it extends essentially outside of the PHBH protein moiety (out) to the conformation in which it is buried within the protein (in) show significantly larger wavenumber shifts (1259/1240 cm^{-1} and 1159/1151 cm^{-1}),[21,24] relative to those observed upon the binding of FMN to Complex I. Thus, the hydrogen bonding to N(3)-H in Complex I is unlikely to be as weak as in the "in" form of PHBH. The RR results appear to be consistent with the reported X-ray structure that indicates that N(3)-H does not have a typical hydrogen bond and is surrounded by a negatively charged surface.[8] This structure suggests that the N(3)-H group is weakly polarized.

In addition to the lower-frequency shift of band X (1258/1252 cm^{-1}) that occurs upon binding of FMN to Complex I from aqueous solution, the high-frequency shift of band II (1584/1587 cm^{-1}), the intensity decrease and/or lower-frequency shift in band IX (1279 cm^{-1}), and the change in the intensity ratio of band III (1550 cm^{-1}) to band IV (1501 cm^{-1}) are consistent with the FMN RR spectral changes detected upon changing the medium from water to dimethyl sulfoxide or acetonitrile.[10,21,23,25] Thus, these results suggest that hydrogen bonds to N(1), N(5), N(3)-H, C(2)=O, and C(4)=O are also weakened in the protein matrix of Complex I (dimethyl sulfoxide functions as a hydrogen bond acceptor and not as a hydrogen bond donor, while acetonitrile is not able to form a hydrogen bond). These changes of medium do not identify the hydrogen bonding sites that induce these RR

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spectral changes. However, the high-frequency shift of band II, which involves C(4a)−N(5) and C(10a)−N(1) stretches, suggests that the hydrogen bonding to N(1) and/or N(5) is weakened.[10,23,25]

The high-dielectric constant environment of the FMN of Complex I as described above is likely to contribute to the weakening of these hydrogen bonds. These weak hydrogen bonds could enhance the adaptability of the isoalloxazine group for various fine controls of the function of the ring.

A Possible Mechanism for Preventing the Spontaneous Production of Reactive Oxygen Species at the FMN Site of Complex I. The C(4a) site of the reduced form of FMN in solution reacts strongly with O_2 to form a peroxide-bound derivative, which is the initial step in the production of various reactive oxygen species. Thus, to avoid spontaneous production of reactive oxygen species during the catalytic turnover of Complex I, we must effectively suppress the reaction of O_2 to C(4a). However, the findings given below indicate that the C(4a) site is not sterically blocked against external O_2 access. (1) The reported X-ray structure of the oxidized and reduced Complex I in the absence of NAD^+ indicates that the C(4a) site is essentially freely accessible to solvent. In fact, the location of the C(4a) site in the X-ray structure[8] suggests that the C(4a) site is more solvent accessible than the N(3)-H group, the solvent accessibility of which has been shown by our previous RR analysis.[12] These results suggest that the C(4a) site is essentially freely solvent accessible during the catalytic turnover. On the other hand, the steady state kinetic analyses for this enzyme system indicate an obligatory order of substrate binding and product release (Q−NADH−NAD^+−QH_2). Namely, NAD^+ is released before the release of reduced ubiquinone.[26] This suggests that NAD^+ is released before oxidation of the fully reduced FMN during catalytic turnover. Thus, the results obtained under the steady state turnover conditions, together with the reported X-ray structures, strongly suggest that the C(4a) site in the fully reduced state of FMN is solvent accessible at least for some period during catalytic turnover.[26] (2) The reported X-ray structure of the flavin site of the NADH-bound fully reduced Complex I indicates that bound NADH effectively blocks the accessibility of solvent to C(4a) in the reduced state.[8] However, the obligatory order of substrate binding and product release (Q−NADH−NAD^+−QH_2) indicates that NADH reacts with Complex I only once during each catalytic turnover. Thus, the NADH-bound form of fully reduced Complex I is not involved in catalytic turnover. On the other hand, an investigation of single-turnover electron transfer from NADH to fully oxidized Complex I suggests that NAD^+ release is slower than the transfer of the electron from FMN to the iron−sulfur sites.[27] However, the steady state kinetic analyses described above indicate that NADH reacts with the oxidized Complex I after the binding of Q.[26] In other words, the reaction process between NADH and oxidized Complex I free from Q is not involved in catalytic turnover. Thus, the single-turnover results cannot exclude the possibility that the solvent accessible FMN in the fully reduced state appears under steady state turnover conditions, as described above. These findings strongly suggest that some nonsterical control system for the reactivity of the C(4a) site to O_2 by the protein moiety of Complex I is facilitated for minimal production of reactive oxygen species.

The small but significant wavenumber shift in band I (1631/1633 cm^{-1}) and the significant increase in the intensity of the 1252 cm^{-1} band upon binding of FMN to Complex I suggest the formation of a hydrogen bond to C(2)=O.[10,11,22,23] As described above, the N(3)-H group is not hydrogen bonded but weakly polarized. This weak polarization of N(3)-H would stabilize the hydrogen bond to C(2)=O by increasing the polarity of C(2)=O group as shown in Figure 3. It has been

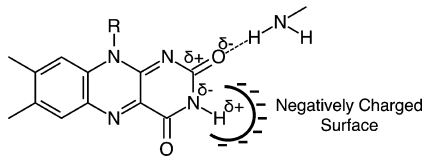

Figure 3. Stabilization of the quinoid form of FMN of Complex I.

proposed that the hydrogen bond to C(2)=O effectively stabilizes the quinoid form (the oxidized form) in the oxidized state.[10,11,22,23] This would lower the redox potential of FMN, which could accelerate the transfer of the electron to the iron−sulfur proteins. The decrease in the redox potential increases the rate of donation of an electron to the iron−sulfur centers of Complex I from the FMN, relative to the electron-accepting rate from NADH. Thus, this could shorten the duration of the fully reduced state of FMN during catalytic turnover to decrease the likelihood of the possibility of exposure of the reduced FMN to O_2. However, strong stabilization of the oxidized form could lower the overall electron transfer rate through FMN to a level that is too slow for normal mitochondrial respiration. Thus, the appropriate (or optimal) strength of the hydrogen bond to C(2)=O for effective electron transfer through the FMN site for giving minimal exposure to O_2 is likely to be maintained by fine-tuning of the polarization of C(2)=O by the N(3)-H group adjacent to C(2)=O.

The reported X-ray structures of the FMN site indicate that the C(2)=O environment is not influenced largely by the oxidation state of Complex I and indicate the presence of a hydrogen bond between the C(2)=O and NH_2 group of Asn220 in both oxidation states.[8] Thus, the C(2)=O group is also fairly polarized in the reduced state as in the oxidized state as suggested by our RR analysis. It has been proposed that modulation of the resonance state of reduced flavin controls the reactivity of the C(4a) site with O_2 as indicated in Figure 4.[28] Thus, polarization of C(2)=O in the reduced state (or formation of a strong hydrogen bond) is expected to favor one of the resonance forms with low electron density at C(4a) (form IV in Figure 4), which would decrease the reactivity of C(4a) with O_2.

As described above, in the oxidized state, the appropriate (or optimal) strength of the hydrogen bond to C(2)=O is necessary for providing effective electron transfer through the FMN site with minimal exposure of the reduced FMN to O_2. On the other hand, in the reduced state, a stronger hydrogen bond to C(2)=O would be preferable to decrease the electron density at C(4a), which is unlikely to significantly influence the redox potential of the isoalloxazine. Thus, the N(3)-H group, located adjacent to C(2)=O, is likely to control the stability of the hydrogen bond to C(2)=O depending on the oxidation state of Complex I. In fact, the X-ray structure of reduced Complex I, in contrast to that of oxidized Complex I, shows the presence of a hydrogen bond to N(3)-H.[8] The redox-coupled X-ray structural change strongly supports the role of N(3)-H for the fine-tuning of the hydrogen bonding to C(2)=O depending on the oxidation state of the FMN for the

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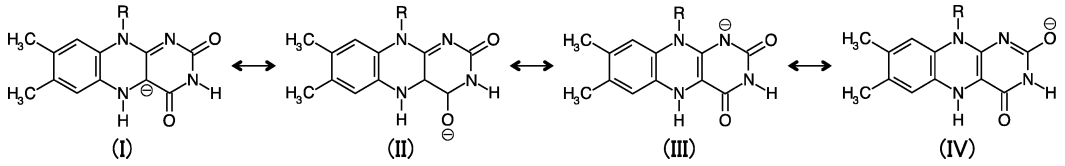

Figure 4. Resonance hybridization of anionic reduced flavin.

prevention of spontaneous production of reactive oxygen species.

The positions of the empirical marker bands for the dielectric environment (bands IV, VI, and VII) and the shifts and band intensity changes in bands X, II, IX, III, and IV, as described above, strongly suggest that all possible hydrogen bonds to the FMN site are likely to be weak. Thus, the hydrogen bond to C(2)═O, even if not very strong, could effectively stabilize the quinoid form (the oxidized form) in the fully oxidized form without significant interference from the other hydrogen bond structures. Possible hydrogen bonds to the fully reduced FMN site are also likely to be weak in the fully reduced state, because essentially no significant redox-coupled X-ray structural change can be detected in the flavin environment of Complex I.[8] Thus, the other hydrogen bond structures would not interfere with the hydrogen bond to C(2)═O that effectively decreases the electron density at C(4a). Furthermore, the high-dielectric constant environment of the FMN of Complex I shown by this RR analysis is expected to interfere with the access of the hydrophobic O_2 molecule to the FMN beyond the dielectric area.

It is well-known that Complex I produces most reactive oxygen species in the mitochondrial respiratory system suffered by nonphysiological perturbation, although the site in Complex I has not been determined. As described above, these RR results suggest that FMN is not tightly fixed in the flavin site. Therefore, the integrity of flavin binding is likely to be sensitive to any nonphysiological perturbation that would impair the fine control system that prevents the binding of O_2 to C(4a). Thus, these RR results suggest that the FMN site is a candidate for the spontaneous production of reactive oxygen species under physiologically harsh conditions.

AUTHOR INFORMATION

Corresponding Author

*E-mail: yoshi@sci.u-hyogo.ac.jp. Phone: 81-791-58-0345.

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ABBREVIATIONS

Complex I, NADH:ubiquinone oxidoreductase; RR, resonance Raman.

REFERENCES

(1) Hatefi, Y., Haavik, A. G., and Jurtshuk, P. (1961) Studies on the electron transport system. XXX. DPNH-cytochrome *c* reductase I. *Biochim. Biophys. Acta 52*, 106−118.

(2) Walker, J. E. (1992) The NADH:ubiquinone oxidoreductase (complex I) of respiratory chains. *Q. Rev. Biophys. 25*, 253−324.

(3) Shinzawa-Itoh, K., Seiyama, J., Terada, H., Nakatsubo, R., Naoki, K., Nakashima, Y., and Yoshikawa, S. (2010) Bovine Heart NADH-Ubiquinone Oxidoreductase Contains One Molecule of Ubiquinone with Ten Isoprene Units as One of the Cofactors. *Biochemistry 49*, 487−492.

(4) Sazanov, L. A., and Hinchliffe, P. (2006) Structure of the Hydrophilic Domain of Respiratory Complex I from *Thermus thermophilus*. *Science 311*, 1430−1436.

(5) Efremov, R. G., Baradaran, R., and Sazanov, L. A. (2010) The architecture of respiratory complex I. *Nature 465*, 441−445.

(6) Efremov, R. G., and Sazanov, L. A. (2011) Structure of the membrane domain of respiratory complex I. *Nature 476*, 414−420.

(7) Hunte, C., Zickermann, V., and Brandt, U. (2010) Functional Modules and Structural Basis of Conformational Coupling in Mitochondrial Complex I. *Science 329*, 448−451.

(8) Berrisford, J. M., and Sazanov, L. A. (2009) Structural Basis for the Mechanism of Respiratory Complex I. *J. Biol. Chem. 284*, 29773−29783.

(9) Bowman, W. D., and Spiro, T. G. (1981) Normal Mode Analysis of Lumiflavin and Interpretation of Resonance Raman Spectra of Flavoproteins. *Biochemistry 20*, 3313−3318.

(10) Li, J., and Kitagawa, T. (2012) Resonance Raman Spectroscopy. In *Methods in Molecular Biology: Flavins and Flavoproteins*, Humana Press, Totowa, NJ.

(11) Eisenberg, A. S., and Schelvis, J. P. M. (2008) Contribution of the 8-methyl group to the vibrational normal modes of flavin mononucleotide and its 5-methyl semiquinone radical. *J. Phys. Chem. A 112*, 6179−6189.

(12) Sugiyama, H., Nakatsubo, R., Yamaguchi, S., Ogura, T., Shinzawa-Itoh, K., and Yoshikawa, S. (2007) Resonance Raman spectra of the FMN of the bovine heart NADH:ubiquinone oxidoreductase, the largest membrane protein in the mitochondrial respiratory system. *J. Bioenerg. Biomembr. 39*, 145−148.

(13) Nishina, Y., Kitagawa, T., Shiga, K., Horiike, K., Matsumura, Y., Watari, H., and Yamano, T. (1978) Resonance Raman Spectra of Riboflavin and Its Derivatives in the Bound State with Egg Riboflavin Binding Proteins. *J. Biochem. 84*, 925−932.

(14) Kitagawa, T., Nishina, Y., Kyogoku, Y., Yamano, T., Ohishi, N., Takai-Suzuki, A., and Yagi, K. (1979) Resonance Raman Spectra of Carbon-13- and Nitrogen-15-Labeled Riboflavin Bound to Egg-White Flavoprotein. *Biochemistry 18*, 1804−1808.

(15) Willis, L. J., and Loehr, T. M. (1985) Resonance Raman Studies of the Flavin and Iron-Sulfur Centers of Milk Xanthine Oxidase. *Biochemistry 24*, 2768−2772.

(16) Desbois, A., Tegoni, M., Gervais, M., and Lutz, M. (1989) Flavin and Heme Structures in Lactate:Cytochrome *c* Oxidoreductase: A Resonance Raman Study. *Biochemistry 28*, 8011−8022.

(17) Yachandra, V. K., Hare, J., Gewirth, A., Czernuszewicz, R. S., Kimura, T., Holm, R. H., and Spiro, T. G. (1983) Resonance Raman

Spectra of Spinach Ferredoxin and Adrenodoxin and of Analogue Complexes. *J. Am. Chem. Soc. 105*, 6462−6468.
(18) Han, S., Czernuszewicz, R. S., Kimura, T., Adams, M. W. W., and Spiro, T. G. (1989) Fe_2S_2 Protein Resonance Raman Spectra Revisited: Structural Variations among Adrenodoxin, Ferredoxin, and Red Paramagnetic Protein. *J. Am. Chem. Soc. 111*, 3505−3511.
(19) Czernuszewicz, R. S., Macor, K. A., Johnson, M. K., Gewirth, A., and Spiro, T. G. (1987) Vibrational Mode Structure and Symmetry in Proteins and Analogues Containing Fe_4S_4 Clusters: Resonance Raman Evidence for Different Degrees of Distortion in HiPIP and Ferredoxin. *J. Am. Chem. Soc. 109*, 7178−7187.
(20) Backes, G., Mino, Y., Loehr, T. M., Meyer, T. E., Cusanovich, M. A., Sweeney, W. V., Adman, E. T., and Sanders-Loehr, J. (1991) The Environment of Fe_4S_4 Clusters in Ferredoxins and High-Potential Iron Proteins. New Information from X-ray Crystallography and Resonance Raman Spectroscopy. *J. Am. Chem. Soc. 113*, 2055−2064.
(21) Zheng, Y., Dong, J., Palfey, B. A., and Carey, P. R. (1999) Using Raman Spectroscopy To Monitor the Solvent-Exposed and "Buried" Forms of Flavin in *p*-Hydroxybenzoate Hydroxylase. *Biochemistry 38*, 16727−16732.
(22) Visser, A. J. W. G., Vervoort, J., O'Kane, D. J., Lee, J., and Carreira, L. A. (1983) Raman Spectra of Flavin Bound in Flavodoxins and in Other Flavoproteins. Evidence for Structural Variations in the Flavin-Binding Region. *Eur. J. Biochem. 131*, 639−645.
(23) Lively, C. R., and McFarland, J. T. (1990) Assignment and the effect of hydrogen bonding on the vibrational normal modes of flavins and flavoproteins. *J. Phys. Chem. 94*, 3980−3994.
(24) Altose, M. D., Zheng, Y., Dong, J., Palfey, B. A., and Carey, P. R. (2001) Comparing protein-ligand interactions in solution and single crystals by Raman spectroscopy. *Proc. Natl. Acad. Sci. U.S.A. 98*, 3006−3011.
(25) Schmidt, J., Coudron, P., Thompson, A. W., Watters, K. L., and McFarland, J. T. (1983) Hydrogen Bonding between Flavin and Protein: A Resonance Raman Study. *Biochemistry 22*, 76−84.
(26) Nakashima, Y., Shinzawa-Itoh, K., Watanabe, K., Naoki, K., Hano, N., and Yoshikawa, S. (2002) The Second Coenzyme Q_1 Binding Site of Bovine Heart NADH:Coenzyme Q Oxidoreductase. *J. Bioenerg. Biomembr. 34*, 89−94.
(27) Verkhovskaya, M. L., Belevich, N., Euro, L., Wikstrom, M., and Verkhovsky, M. I. (2008) Real-time electron transfer in respiratory complex I. *Proc. Natl. Acad. Sci. U.S.A. 105*, 3763−3767.
(28) Miura, R. (2001) Versatility and Specificity in Flavoenzymes: Control Mechanisms of Flavin Reactivity. *Chem. Rec. 1*, 183−194.