Thermodynamic Analysis of Flavin in Mitochondrial NADH:Ubiquinone Oxidoreductase (Complex I)†

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ABSTRACT: This paper reports the first direct characterization of flavin (noncovalently bound FMN) in energy coupling site I of the mitochondrial respiratory chain. Thermodynamic parameters of its redox reactions were determined potentiometrically monitoring the $g = 2.005$ signal of its free radical form in isolated bovine heart NADH:ubiquinone oxidoreductase (complex I). The midpoint redox potentials of consecutive one-electron reduction steps are $E_m^{1/0} = -414$ mV and $E_m^{2/1} = -336$ mV at pH 7.5. This corresponds to a stability constant of the intermediate flavosemiquinone state of 4.5×10^{-2}. The pK values of the free radical ($Fl^{\bullet-} \leftrightarrow FlH^{\bullet}$) and reduced flavin ($FlH^- \leftrightarrow FlH_2$) were estimated as 7.7 and 7.1, respectively. The potentiometrically obtained $g = 2.005$ flavin free radical EPR signal revealed an unusually broad (2.4 mT) and pH-independent peak-to-peak line width. The spin relaxation of flavosemiquinone in complex I is much faster than that of flavodoxin due to strong dipole–dipole interaction with iron–sulfur cluster N3. Guanidine, an activator of NADH–ferricyanide reductase activity of complex I, was found to have a strong stabilizing effect on the flavin free radical generated both by equilibration with the NADH/NAD^+ redox couple and by potentiometric redox titration. The addition of guanidine also leads to a slight modification of the EPR spectrum of iron–sulfur cluster N3. Anaerobic titration of flavosemiquinone free radical with the strictly $n = 2$ NADH/NAD^+ and APADH/$APAD^+$ redox couples revealed that nucleotide binding narrows the EPR signal line width of the flavin free radical to 1.7 mT and changes a shape of the titration curve. This suggests a conformational change of the complex due to the substrate binding.

The NADH:ubiquinone oxidoreductase (complex I) from mammalian mitochondria consists of 42 different subunits [Fearnley & Walker, 1992; Walker, 1992], and as electron carriers it contains noncovalently bound FMN[1] [Rao *et al.*, 1963], at least two forms of bound ubiquinone [Hatefi, 1978; Suzuki & King, 1983; Burbaev *et al.*, 1989], and six distinct EPR-detectable iron–sulfur clusters [Ohnishi, 1979; Beinert & Albracht, 1982; Ohnishi & Salerno, 1982, Ohnishi, 1993], although the existence of additional EPR-silent clusters cannot be ruled out [Ragan, 1987; Hatefi, 1985]. The iron content [22–24 atom/FMN (Ragan *et al.*, 1982)] and the number of highly conserved cysteine-rich motifs, which are revealed from the primary sequences of the subunits of complex I [Fearnley & Walker, 1992], allow the ligation of up to eight iron–sulfur clusters. Complex I catalyzes the oxidation of NADH by ubiquinone coupled to proton translation across the inner membrane, but the path of electron flow through the intrinsic redox components of the enzyme and the mechanism of energy coupling are unknown.

The sequence of electron-transfer steps among the redox components of complex I is not amenable to direct kinetic analysis, because electron equilibration between them occurs faster than electron entry from NADH to the enzyme. According to the potentiometrically determined midpoint redox potential values (E_m), the iron–sulfur clusters can be divided into three groups: low-potential N1a ($E_{m,7.0} = -380$ in bovine heart submitochondrial particles and <-500 to -380 mV in different preparations of complex I), a group of isopotential clusters N1b, N3, N4, and N5 ($E_{m,7.0} = -240$ to -270 mV), and high-potential N2 ($E_{m,7.0} = -50$ to -120 mV) [Ohnishi, 1979; Ingledew & Ohnishi, 1980]. Only the E_m of N1a and N2 were found to be dependent on pH and phosphorylation potential, indicating their possible involvement in proton pumping [Ohnishi, 1976; DeVault, 1976].

A rather high 3–5 $H^+/2e^-$ stoichiometry at this site [Rottenberg & Gutman, 1977; Brown & Brandt, 1988; Lemasters, 1984; Scholes & Hinkle, 1984; Wikström, 1984] cannot be explained by a simple chemiosmotic loop mechanism, as originally proposed by Mitchell (1966). Hence, various cyclic schemes have been proposed which include both flavin and ubiquinone as specific transmembraneous proton carriers [Suzuki & King, 1983; Ragan, 1987, 1990; Krishnamoorthy & Hinkle, 1988; Kotlyar *et al.*, 1990; Weiss *et al.*, 1991]. However, to date there has been no hard experimental information about the role of the FMN molecule in energy transduction nor about its physicochemical properties and localization in complex I.

It has been generally believed that flavin is most likely the immediate oxidant of NADH, as in the case of many other flavin-containing dehydrogenases [Mathews, 1991]. Since the 51-kDa subunit was shown to bind NADH [Chen & Guillory, 1981, 1984], the flavin binding site was considered

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[1] Abbreviations: FMN, flavin mononucleotide, APADH, 3-acetylpyridine adenine dinucleotide, reduced form; Bis-Tris-propane, 1,3-bis[tris(hydroxymethyl)methylamino]propane; Hepes, 4-(2-hydroxymethyl)-1-piperazineethanesulfonic acid; MES, 4-morpholineethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid; E_h, ambient redox potential either measured directly or calculated from the poise of the substrate couple; E_m, midpoint redox potential; $E_{m,7.0}$, midpoint redox potential at pH 7.0; EPR, electron paramagnetic resonance.

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to be in the same subunit. The conserved region between amino acids 180 and 226 in the primary sequence of the bovine heart 51-kDa polypeptide was proposed to be responsible for flavin binding [Pilkington *et al.*, 1991]. Most recently, Fecke *et al.* (1994) specifically disrupted the 51-kDa subunit of *Neurospora crassa* complex I by homologous replacement with a defective gene copy. This *nuo*51 mutant complex I is assembled almost entirely except for the NADH binding 51-kDa subunit. It completely lacks NADH–ferricyanide and NADH–Q_2 oxidoreductase activities, flavin, and iron–sulfur cluster N3 (all other iron–sulfur clusters are present). This information provides the strongest evidence for the notion that flavin is localized in the 51-kDa NADH binding subunit. The flavoprotein (FP) subcomplex of the enzyme, which is capable of catalyzing NADH oxidation by artificial electron acceptors [Galante & Hatefi, 1978; Hatefi & Hanstein, 1975], is composed of only the 51-, 24-, and 9-kDa polypeptides, which is consistent with the above-proposed 51-kDa subunit localization of the flavin.

In order to understand the role of flavin in electron transfer and in the putative redox-driven proton-pumping mechanism in coupling site I, it is essential to know its thermodynamic and kinetic properties. Spectrophotometric measurements of FMN in the visible region are obscured by the contribution from iron–sulfur clusters; therefore, EPR is currently the method of choice for monitoring the flavin free radical. In the present work, we report the first detailed analyses of the potentiometric and EPR characterization of flavin in isolated bovine heart complex I, discuss its role as a converter from $n = 2$ (NADH) to $n = 1$ (iron–sulfur clusters) electron transfer steps, and offer speculations regarding the possible mechanisms of flavin participation in proton translocation.

MATERIALS AND METHODS

Complex I was prepared from bovine heart mitochondria as described earlier [Hatefi, 1978]. Potentiometric titration of complex I was conducted anaerobically according to Dutton (1978). In order to minimize interference with the $g = 2.00$ signal from redox mediator dyes, the following selected redox dyes were used: pyocyanin, indigo tetrasulfonate, indigo trisulfonate, indigo disulfonate, 2-hydroxy-1,4-naphthoquinone, 3-methyl-2-hydroxy-1,4-naphthoquinone, phenosafranin, safranin T, and neutral red, each at a final concentration of 40 μM. The contribution of dye signals was further minimized by specifically saturating them using high microwave powers (100 mW) since the FMN free radical in complex I exhibits very fast spin relaxation. This is especially important at pH < 7, where safranin dyes elicit a noticeable free radical EPR signal [Michaelis, 1936]. Reaction media contained 50 mM MES, MOPS, Bis-Tris-propane, or Hepes (depending on the pH), 0.67 M sucrose, 0.5 mM EDTA, and 0.05% sodium deoxycholate. The pH of the enzyme suspension was checked before and after the titration; the shift in pH value after the addition of considerable amounts of dithionite never exceeded 0.2 unit.

The titrations with the substrate couples (NADH/NAD^+ and APADH/$APAD^+$) were performed anaerobically under an argon atmosphere; a separate incubation was performed for each E_h value, equilibrating the system for 15 min at room temperature. The concentration of individual nucleotides was varied from 100 μM to 5 mM while keeping the total concentration approximately constant at 5 mM. Other additions are documented in the figure legends. EPR samples were rapidly frozen in a cold isopentane:methylcyclohexane (5:1) freezing mixture and stored in liquid nitrogen until examination.

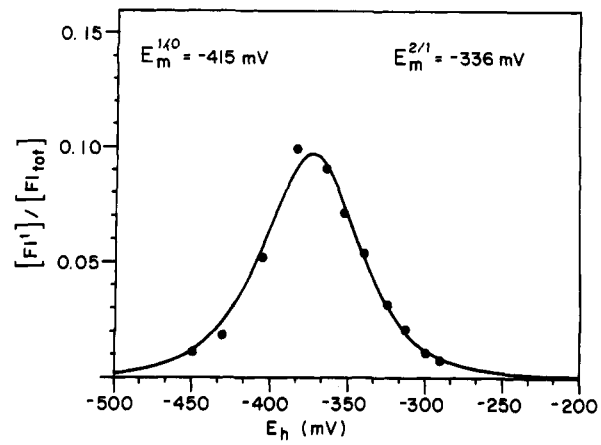

FIGURE 1: Potentiometric titration of the flavin free radical EPR signal ($g = 2.005$) in bovine heart complex I. The enzyme (5.8 μM flavin concentration) was titrated anaerobically in 50 mM Hepes (pH 7.5). Other components of the medium and redox dyes are described in Materials and Methods. EPR conditions: microwave power, 100 mW; modulation amplitude, 0.8 mT; microwave frequency, 9.1 GHz; time constant, 0.128 s; sample temperature, 169 K. The best fit theoretical titration curve is drawn through the experimental points. The maximum spin concentration (I_{max}) of free radical was obtained from spin quantitation of the signal as described in Materials and Methods.

EPR measurements were performed with a Varian-E109 spectrometer operating at X-band (9.2 GHz). The temperature of the sample was controlled by a variable temperature helium flow cryostat (Air Products, Model LTD-3-110) for the temperature range between 5 and 50 K. For the higher temperature range, we used a Varian liquid nitrogen flow system in which temperature was regulated by the Johnson Foundation's digital temperature controller.

Spin quantitation of the free radical signals was conducted by double integration of the $g = 2.00$ signal using a flavodoxin free radical from *Megasphera elsdenii* [Massey & Palmer, 1966] or the spin label 3-(maleimidomethyl)proxyl as standards under nonpower saturated conditions, typically at 50 μW for the standard and at 20–50 mW for complex I flavin at 173 K. Measurements of the relative reduction levels of the iron–sulfur clusters in complex I were performed according to Ohnishi (1979) and Beinert and Albracht (1982); cluster N1b was estimated from the peak-to-peak amplitude of the $g = 1.94$ signal at 40 K; N2 was estimated from the peak height of the $g = 2.054$ signal at 14 K; N3 and N4 were estimated from the peak heights of the $g_x = 1.86$ and $g_z = 2.10$ signals at 8 K, respectively.

All computer analyses of theoretical best fit curves were conducted utilizing GIM (Graphic Interactive Management) software from Drachev Development (1522 E. Southern, No. 2016, Tempe, AZ 85282).

The flavin content in complex I preparations was determined fluorometrically [Faeder & Siegel, 1973]. Protein concentration was determined using the biuret method [Gornall *et al.*, 1949].

RESULTS

Figure 1 presents a potentiometric titration of the flavin free radical in complex I at pH 7.5. The relative peak-to-peak amplitude of the $g = 2.005$ EPR signals was plotted as a function of the ambient E_h values. The Y-axis scale was converted to the relative flavosemiquinone concentration ($[Fl^1]/[Fl_{tot}]$) which was determined from spin quantitation of the $g = 2.005$ EPR signal and from the total FMN concentration in this preparation. In separate experiments, we have shown that neither rotenone nor piericidin A affects

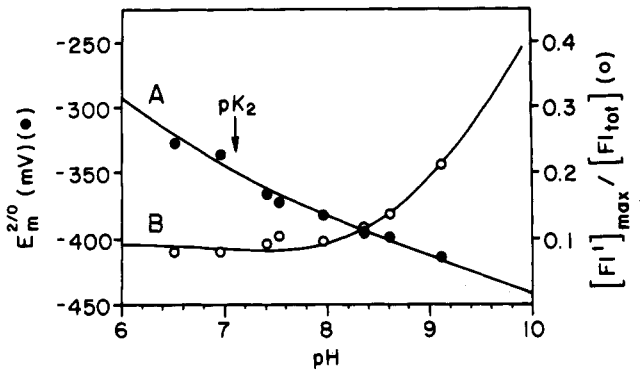

FIGURE 2: pH dependence of the midpoint redox potential of the two-electron flavin reduction ($E_m^{2/0}$) (A) and of the maximum free radical concentration from potentiometric titration (B).

the titration profile or the line shape of the observed free radical signal (not shown).

We define the midpoint redox potentials of two consecutive one-electron transfer steps ($Fl^0 \leftrightarrow Fl^1 \leftrightarrow Fl^2$) as $E_m^{1/0}$ and $E_m^{2/1}$, respectively. Theoretically, the maximum E_h position of the bell-shaped titration curve corresponds to a midpoint redox potential $E_m^{2/0}$ of the overall two-electron transfer reaction ($Fl^0 \leftrightarrow Fl^2$) which is equal to $(E_m^{1/0} + E_m^{2/1})/2$, while the maximal relative concentration of semireduced flavin [Fl^1] is predetermined by the difference of the midpoint potentials of the individual one-electron transfer steps, which can be related with the free radical stability constant (K) as $E_m^{1/0} - E_m^{2/1} = 2.3RT/F \log K$; where R is the universal gas constant, F is the Faraday constant, and T is the absolute temperature. When the temperature is 25 °C, the factor $2.3RT/F$ becomes 59 (mV). Dependence of the relative concentration of the intermediate semireduced form (Fl^1/Fl_{tot}) on the ambient redox potential (E_h) is described by the following equation [Clark, 1960]:

$$Fl^1/Fl_{tot} = \frac{1}{1 + 10^{(E_h - E_m^{1/0})/59} + 10^{(E_m^{2/1} - E_h)/59}} \quad (1)$$

For the titration data analysis, as exemplified in Figure 1, the best fit theoretical curve corresponding to eq 1 was drawn with simulated $E_{m,7.5}^{1/0}$ and $E_{m,7.5}^{2/1}$ values of −415 and −336 mV, respectively. This corresponds to a stability constant of the intermediate flavosemiquinone state of 4.5×10^{-2}. The relatively high stability of the flavin free radical state in complex I ($K = 3.4 \times 10^{-2}$ at pH 7.0 as shown later) suggests that the flavin itself may function as a converter from $n = 2$ (NADH) to $n = 1$ (iron–sulfur clusters) as in the case of flavin in succinate dehydrogenase ($K = 2.5 \times 10^{-2}$ at pH 7.0) [Ohnishi *et al.*, 1981b; Hederstedt & Ohnishi, 1992] and Q_i in the mitochondrial cytochrome bc_1 complex ($K = 5 \times 10^{-2}$ at pH 7.0) [deVries *et al.*, 1980; Bowyer & Ohnishi, 1985].

In order to analyze the protonation states of flavin in complex I, we have undertaken titration of the enzyme at different pH values. Figure 2 shows the pH dependence of the $E_m^{2/0}$ for the overall two-electron transfer (curve A) and of the maximal relative flavosemiquinone concentration (curve B), determined directly from the free radical titration curves at different pH values. The straight line with a slope of −30 mV/pH, which fits the pH dependence of $E_m^{2/0}$ at values above pH 8.0, indicates the involvement of only one proton in the two-electron flavin reduction ($Fl \leftrightarrow FlH^-$) under these conditions. Below pH 7.5, maximal flavin free radical concentration remains constant, and one can see flavosemiquinone stabilization at

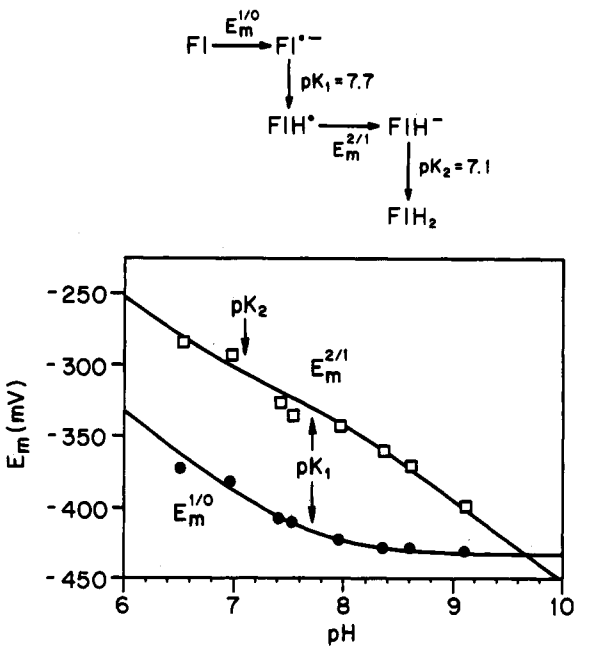

FIGURE 3: pH dependence of the midpoint redox potentials of two one-electron transfer steps ($E_m^{1/0}$ and $E_m^{2/1}$) of flavin in complex I. Individual $E_m^{1/0}$ and $E_m^{2/1}$ at different pH values were calculated from the best fit titration curves exemplified in Figure 1.

pH > 7.5. Computer analysis of the pH dependencies of E_m values for the consecutive one-electron transfer reactions ($E_m^{1/0}$ and $E_m^{2/1}$) is presented in Figure 3. Fitting the theoretical curves gave values of 7.7 and 7.1 for pK_1 ($Fl^{\bullet -} \leftrightarrow FlH^\bullet$ and pK_2 ($FlH^- \leftrightarrow FlH_2$), respectively. Thus, at pH 8.7, 90% of the flavosemiquinone (Fl^1) should be in the anionic form ($Fl^{\bullet -}$). On the other hand, the potentiometrically obtained $g = 2.00$ flavin free radical EPR signal of complex I revealed an unusually broad (2.4 mT) and pH-independent peak-to-peak line width (Figure 4A,B), which is even wider than that of the neutral protonated free radical species (1.8–2.0 mT) of various flavoproteins [Massey & Palmer, 1966; Palmer *et al.*, 1971]. In addition, the EPR signal of the flavin free radical does not show Curie law temperature dependence: signal amplitude decreased (most probably due to broadening) with lowering of the sample temperature under the temperature range studied (from 200 to 77 K), with almost no changes in spin relaxation characteristics (Figure 5, curves B and C). The nature of this behavior is not understood and is currently under investigation. pH-independent flavin free radical signal line width may be explained by the protonation of a neighboring amino acid residue of the complex I concurrent with the reduction of the flavin as it takes place in the photosynthetic reaction center primary quinone acceptor Q_A case [see Okamura and Feher (1992) and references cited therein] or in the succinate dehydrogenase flavosemiquinone case [Edmondson *et al.*, 1981; Williamson & Edmondson, 1985]. On the other hand, since the spin relaxation of flavosemiquinone in complex I is much faster than that of flavodoxin, which contains no transition metal ions in the vicinity of the flavin (Figure 5, curve A), an extraordinary broad line width could originate from strong dipole–dipole interaction between flavosemiquinone and the fast relaxing tetranuclear iron–sulfur cluster N3, as has been suggested earlier from the analysis of the dip in the N3 redox titration and from the observation of a half-field triplet ("$\Delta M_s = 2$") signal in complex I [Salerno *et al.*, 1977; Ingledew & Ohnishi, 1980], therefore it seems quite possible that the peak-to-peak line width change due to protonation of the semiquinone might be masked by this

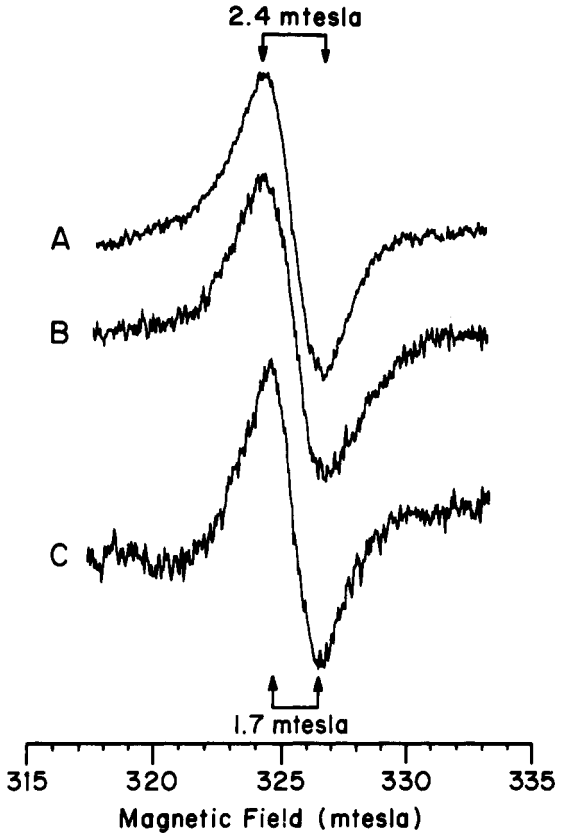

FIGURE 4: EPR spectra of the flavin free radical in complex I. In A and B, the enzyme was potentiometrically poised at −328 and −437 mV, respectively, at pH 6.5 and 9.1. In C, complex I was equilibrated anaerobically with the NADH/NAD^+ couple at pH 8.2. Calculated E_h = −310 mV. EPR conditions as in Figure 1.

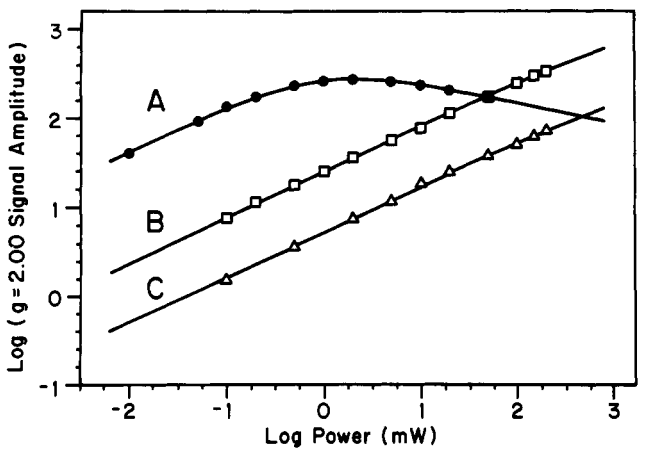

FIGURE 5: Power saturation behavior of flavodoxin (A) and potentiometrically obtained (E_h = −410 mV) flavin free radicals in complex I (pH 8.2) (B and C). EPR conditions as in Figure 1, except the sample temperatures were 150 (curve A), 201 (curve B), and 77 K (curve C).

broadening. Figure 6 compares titration profiles of the g = 2.00 free radical signal amplitudes (Figure 6A) and the g = 1.86 g_x negative peak heights of the cluster N3 (Figure 6B). The trough in the titration curve of the N3 center caused by the broadening of the iron–sulfur EPR signal due to interaction with the free radical coincides with the maximum flavin free radical concentration. Such a coincidence makes it possible to exclude the previously suggested alternative possibility of redox cooperativity between N3 and flavin [Ohnishi & Salerno, 1982]. The same trough on the N3 titration curve (Figure 6B) could be explained by assuming that the midpoint potential

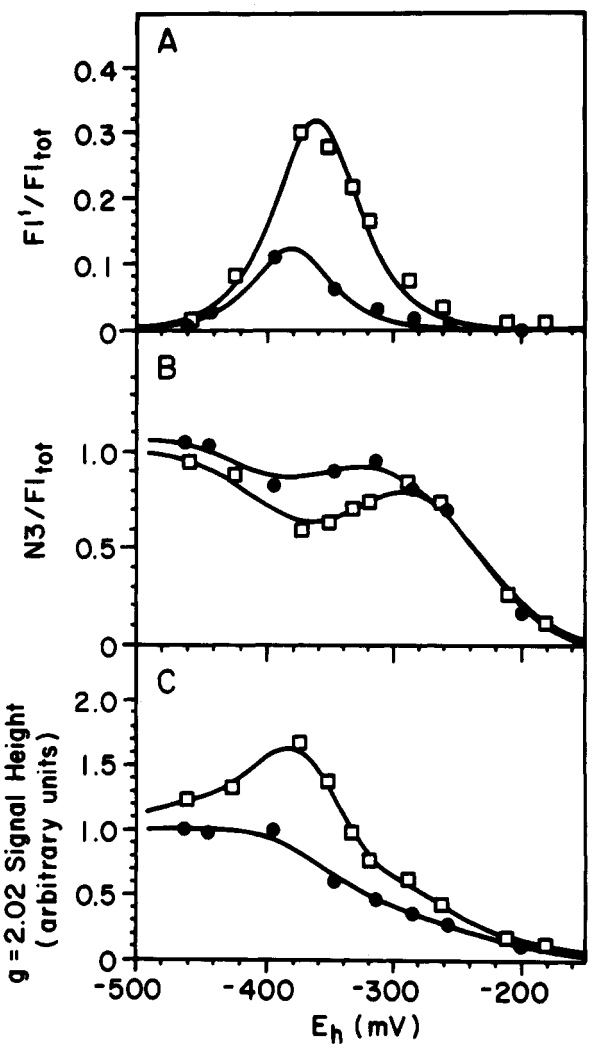

FIGURE 6: Potentiometric redox titration of flavin free radical (A), iron–sulfur cluster N3 (B), and low-temperature g = 2.02 EPR signal (C) in complex I (pH 8.0) in the absence (●) and in the presence (□) of 20 mM guanidine hydrochloride. EPR conditions for (A) as indicated in Figure 1; for (B) and (C) as follows: microwave power, 50 mW; modulation amplitude, 0.8 mT; microwave frequency, 9.25 GHz; time constant, 0.064 s; sample temperature, 9.1 K.

of the N3 iron–sulfur cluster is becoming more negative due to redox interaction with fully reduced flavin. However, in that case one would predict a decrease of the trough depth with an increase of the maximal free radical concentration, and one would not expect the quantitative coincidence observed in the experiment (Figure 6A,B).

Among the few effectors (activators and inhibitors) with a reaction site localized at the donor side of complex I, guanidine deserves special attention for the study of the flavin free radical. Guanidinium compounds were shown to greatly increase the oxidation rate of NADH by ferricyanide and other artificial acceptors as catalyzed by complex I and its soluble FP fragment [Hatefi *et al.*, 1969; Hatefi & Galante, 1977; Galante & Hatefi, 1979]. As seen in Figure 6A, the addition of 20 mM guanidine leads to a considerable stabilization of the flavin free radical. The analysis of the flavosemiquinone titration curves revealed that the addition of 20 mM Guanidine causes an increase of the $E_m^{1/0}$ of the first electron transfer step by 50 mV and a small (11 mV) decrease of the $E_m^{2/1}$ and, therefore, leads to a more than 10-fold elevation of the semiquinone stability constant, from 7.4×10^{-2} to 8.1×10^{-1}. In other words, guanidine preferentially binds to the enzyme having flavin in semireduced

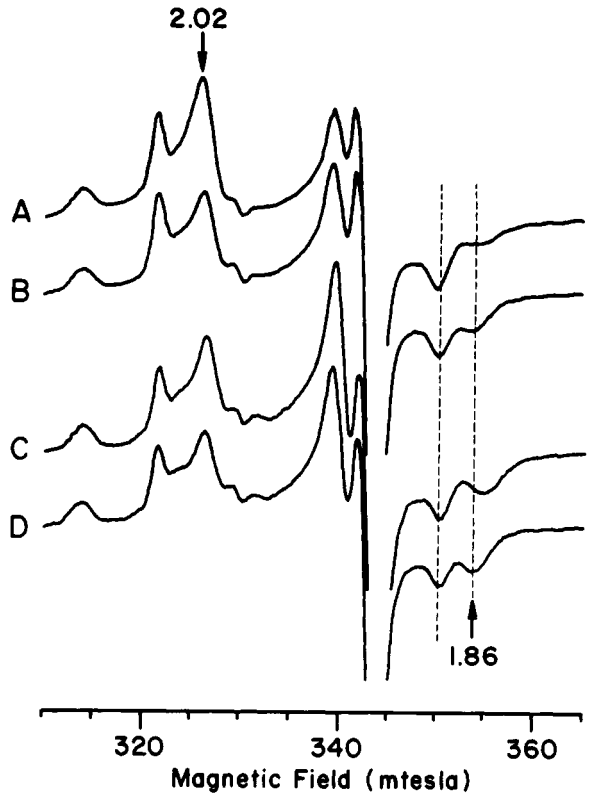

FIGURE 7: Low-temperature EPR spectra of complex I (pH 8.0) potentiometrically poised at E_h −373 (A), −394 (B), −458 (C), and −453 mV (D) in the absence of (B and D) and in the presence (A and C) of 20 mM guanidine hydrochloride. EPR conditions are as in Figure 6 (B and C). All spectra were recorded at the same gain.

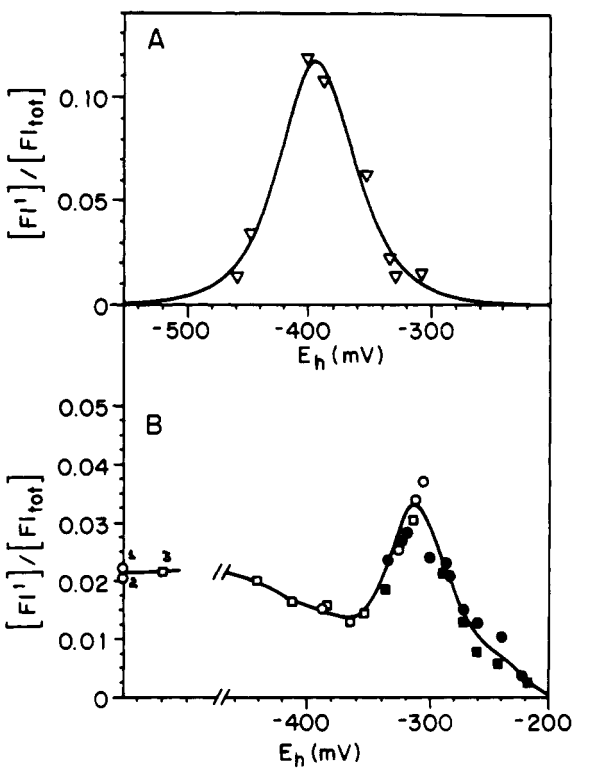

FIGURE 8: Titration of the flavin free radical in complex I (pH 8.2) obtained potentiometrically (A) and by equilibration with NADH/NAD$^+$ (open symbols) and APADH/APAD$^+$ (closed symbols) redox couples (B). Numbered signs correspond to the free radical level after reduction of complex I (11.5 μM total flavin) with NADH (1.3 mM) or APADH (3.8 mM) in the presence of the following NADH-regenerating enzyme systems: (1) ethanol (0.1%), alcohol dehydrogenase from bakers yeast (150 units/mL) and semicarbazide hydrochloride (10 mM); (2) galactose (20 mM) and galactose dehydrogenase from *Pseudomonas fluorescence* (3 units/mL), rough estimation of E_h values assuming six electron equivalent capacity of complex I gives a value of −520 mV; (3) glucose (20 mM) and glucose dehydrogenase from *Bacillus megaterium* (64 units/mL), calculated E_h = −440 mV.

and fully reduced forms. Similar enhancement of flavosemiquinone concentration has also been observed when complex I was titrated with a NADH/NAD$^+$ couple (not shown). The spectral shape and line width of the g = 2.00 signal were unaltered by guanidine. At the same time, the addition of guanidine caused a noticeable shift of g_x in the N3 EPR spectrum to a higher field (Figure 7). This change does not appear to be due to the spin–spin interaction with flavosemiquinone because the same shift could be observed when flavin was in the fully reduced diamagnetic state (Figure 7C,D). Another peculiarity of the effect of guanidine on complex I concerns the appearance of a remarkably fast relaxing g = 2.02 resonance in the EPR spectrum at low temperature (9.1 K) (Figure 7A), which strongly correlates with free radical formation (Figure 6A,C). This signal may be associated with spin coupling between flavosemiquinone and N3 spins; this topic is currently under investigation. It should be mentioned that guanidine has no significant effect on either the EPR spectra or the thermodynamic properties of other iron–sulfur clusters in complex I (N1, N2, and N4).

The anaerobic titrations of the flavosemiquinone free radical by NADH/NAD$^+$ and APADH/APAD$^+$, which are obligatory two-electron equivalent redox couples with $E_{m,8.0}$ = −350 and −278 mV, respectively [Clark, 1960], revealed a series of remarkable differences from potentiometric data. (i) The g = 2.00 EPR signal line width was narrower (1.7 mT) in the presence of a natural electron donor, NADH, as shown earlier (Figure 4C), which suggests following two possibilities: (1) the effect of nucleotide binding on the delocalization of the unpaired electron density or (2) ligand (NADH or APADH) induced conformational change of the protein, which would lead to the receding of the FMN moiety from the N3 cluster, thus weakening their magnetic interaction. Very recently, the substrate-induced conformation changes of complex I have been demonstrated by cross-linking studies [Belogrudov & Hatefi, 1994]. On the other hand, NADH does not change the relaxation characteristics of the flavin free radical (not shown). The flavosemiquinone titration curve by substrate couple (Figure 8B) is very different from that obtained potentiometrically (Figure 8A) at the same pH. (ii) The maximum position of the free radical titration curve is shifted by about 80 mV to a higher potential (−310 mV), and the highest flavosemiquinone concentration achieved by complex I titration with substrate couple is only about 3% of the total flavin concentration, which is approximately 4 times smaller than that obtained potentiometrically (Figure 8A). (iii) Almost 70% of the maximal signal is observable even at the highest reduction level of complex I obtainable by NADH in the presence of NADH-regenerating enzyme system (e.g., ethanol plus alcohol dehydrogenase, glucose plus glucose dehydrogenase, or galactose plus galactose dehydrogenase).

DISCUSSION

As has been shown in the preceding section, the midpoint potential of the two-electron reduction ($E_{m,7.0}^{2/0}$) of FMN in complex I is −340 mV (see Figures 1 and 2), which is 133 mV more negative than the $E_{m,7.0}^{2/0}$ for free FMN (−207 mV) [Draper & Ingraham, 1968]. It follows that the oxidized FMN has more than 4 orders of magnitude higher affinity for

its specific binding site than $FMNH_2$. This possibility is in agreement with the recent finding that $FMNH_2$ tends to dissociate after reduction of the soluble FP fragment of complex I by NADH or dithionite [Sled & Vinogradov, 1993].

Although the relatively high stability constant for the flavosemiquinone in complex I suggests its role as a converter between $n = 2$ and $n = 1$ electron transport steps by itself, the very low (−387 mV at pH 7) value for $E_m^{1/0}$ of the first flavin reduction step (Figure 3) could also provide a possible explanation for participation of the low-potential N1a iron–sulfur cluster in the $FMNH_2$ oxidation reactions. The existence of a flavin free radical after "complete" reduction of complex I by NADH in the presence of NADH-regenerating enzyme systems (Figure 8B) together with the fact that the N1a center is not reducible with NADH in spite of the thermodynamic possibility of this reaction [Ingledew & Ohnishi, 1980; Ohnishi *et al.*, 1981a] may indicate an odd number electron capacity of complex I, so that after the complete reduction of the enzyme by NADH (an obligatory two-electron donor) the last couple of reducing equivalents provided by NADH should equilibrate between the $FMNH_2$/$FMNH^\bullet$ and the N1a(red)/N1a(ox) redox couples. A 2% level of the flavosemiquinone stabilized after this equilibration (Figure 8B), corresponding to the ~100-mV electron energy gap between these two redox couples. Taking E_m of N1a as −500 mV, one could expect $E_m \sim -400$ mV for the $FMNH_2$/$FMNH^\bullet$ redox couple in complex I having the substrate binding site occupied with NADH, if we assume that complex I is a homogeneous protein complex. However, another possible explanation arising from the heterogeneity of complex I preparations cannot be ruled out. The same titration curve (Figure 8B) may also be expected if one assumes that complex I contains an even number of FeS centers, but in ~2% of the enzyme molecules one of the clusters has been lost or has become nonreducible by NADH due to deterioration.

The pK values for the reduced forms of flavin in complex I determined in this work are difficult to reconcile with the proposed mechanisms involving proton translocation by flavin in complex I [Ragan, 1987, 1990; Krishnamoorthy & Hinkle, 1988]. If the pK values obtained do reflect changes in the protonation state of the flavin molecule, then the transfer of a hydride ion from NADH to FMN in complex I at a pH higher than the pK for fully reduced flavin (pK = 7.1) would lead to the production of only the anionic $FMNH^-$ form without absorbing a proton from the matrix side. Whatever scheme of proton transfer we examine [Ragan, 1987, 1990; Krishnamoorthy & Hinkle, 1988] would lead to a drastic decrease of the energetic efficiency of the proton-pumping machinery. A possible solution to this problem could arise through a change of the thermodynamic properties of flavin caused by NADH binding. Taking into account the vast differences in NADH and NAD^+ affinities for the oxidized and reduced complex I, as determined by kinetic methods [Avraam & Kotlyar, 1991], this explanation seems quite possible. Much higher affinity of NADH to the oxidized complex I than to its reduced form (apparent affinities were 0.2 versus 80 μM, respectively) would result in a negative shift in the midpoint potential of a component responsible for the change in nucleotide affinity. The effect of NADH on the flavin free radical EPR signal line width and the differences in the flavin redox titration by the NADH/NAD^+ couple from those obtained potentiometrically could also be considered as an indication of such a change. Another possibility to avoid these difficulties can be provided by assuming the functioning of a strictly redox-linked proton pump on the donor side of complex I [DeVault,

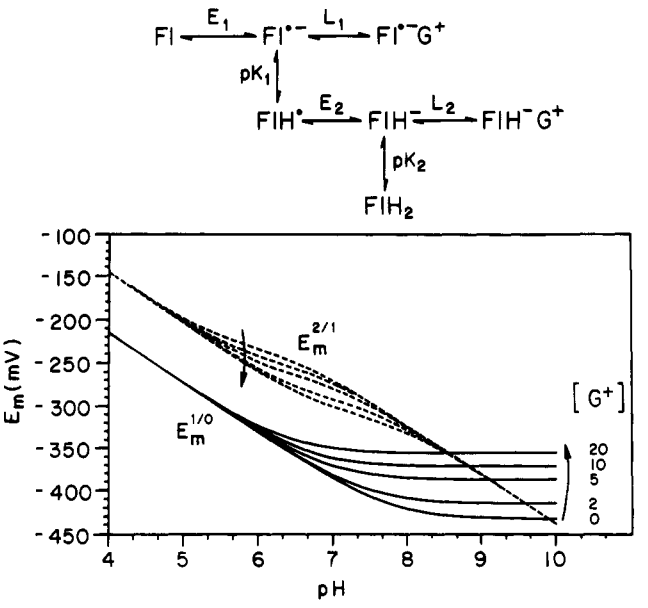

FIGURE 9: Theoretical dependencies of $E_m^{1/0}$ (solid lines) and $E_m^{2/1}$ (dashed lines) on pH in the presence of different concentrations of guanidine (G^+). E_m values were calculated using eqs 2 and 3 and assuming the binding of a positively charged molecule of guanidine (G^+) to anionic forms of flavin ($Fl^{\bullet-}$ and FlH^-) and pseudo-first-order equilibrium constants ($L_1[G^+] = L_2[G^+]$) varying from 0 to 20. Equilibrium constants for transitions between different redox and protonation states of flavin (E_1, E_2, pK_1, and pK_2) were taken as determined above (see Figure 3). Arrows indicate increasing guanidine concentrations.

1971; Wikström & Krab, 1979], i.e., when transmembrane proton pumping is an obligatory condition for electron transfer through the coupling site (i.e., when electron transfer through the coupling site is forbidden without transmembrane proton translocation). If the proton-translocating reaction(s) is not a rate-limiting step in the overall electron transfer through complex I, one can expect a pH-independent H^+/e^- stoichiometry under a wide range of pH without significant changes in the rate of electron transfer. Such a mechanism would result in the absence of sensitivity of the electron transport rate through this coupling site (or subsite) to the applied electrochemical proton gradient (i.e., lack of the respiratory control). We have observed that guanidine induces more than 1 order of magnitude stabilization of the flavin free radical in complex I (Figure 6A). A simple explanation of that could be based on the assumption that guanidine, being a positively charged molecule, preferentially interacts with the anionic forms of flavin (see scheme in Figure 9). From this scheme, the expressions for $E_m^{1/0}$ and $E_m^{2/1}$ in the presence of guanidine can be written as follows:

$$E_m^{1/0} = E_1 + 59 \log (1 + 10^{pK_1-pH} + L_1[G^+]) \quad (2)$$

$$E_m^{2/1} = E_2 + 59 \log \frac{1 + 10^{pK_2-pH} + L_2[G^+]}{1 + 10^{pK_1-pH} + L_1[G^+]} \quad (3)$$

where E_1 and E_2 are the midpoint potentials of $Fl^{\bullet-}/Fl$ and $FlH^-/FlH^\bullet$ redox couples; $[G^+]$ is the concentration of guanidine; L_1 and L_2 are the binding constants of guanidinium cation to complex I that carry flavin in the semireduced and fully reduced anionic forms, respectively. As seen from the theoretical dependencies of $E_m^{1/0}$ and $E_m^{2/1}$ on pH (Figure 9) predicted by eqs 2 and 3, an increase of guanidine concentration will lead to a shift of the pK values of both flavosemiquinone and fully reduced forms to a more acidic

pH. If the affinities of guanidine to the complex I having anionic deprotonated forms of semiquinone ($Fl^{\bullet -}$) and of fully reduced flavin (FlH^-) are approximately equal, the apparent negative shift of both pK values will be the same. At a pH > pK_1 of semiquinone (for example, at pH 8, as shown in Figure 6) one would see a considerable increaes in $E_m^{1/0}$ with a small decrease in $E_m^{2/1}$, which results in the stabilization of flavin semiquinone. The effect of guanidinium compounds on different complex I activities has been extensively studied in early papers by Hatefi and his co-workers [Hatefi *et al.*, 1969; Hatefi & Galante, 1977; Galante & Hatefi, 1979]. The authors demonstrated that the addition of guanidine leads to both an increase in V_{max} and a decrease in K_m for NADH for all of the reactions studied. In the present work we have demonstrated an additional guanidine effect, stabilization of the flavosemiquinone state. Perhaps guanidine binds to a complex I protein site that is close to both NADH and flavin binding sites. The combination of two guanidine effects, namely, substrate affinity increase and flavosemiquinone stabilization, would explain the concurrent increase of substrate affinity and V_{max} of NADH–$K_3Fe(CN)_6$ and some other acceptor reductase activities of complex I.

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