Thermodynamic Analysis of the Binding of Oxidized and Reduced FMN Cofactor to *Vibrio harveyi* NADPH-FMN Oxidoreductase FRP Apoenzyme†

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ABSTRACT: The *Vibrio harveyi* NADPH-specific flavin reductase FRP follows a ping-pong mechanism but switches to a sequential mechanism in the luciferase-coupled reaction. The bound FMN co-isolated with FRP, while acting as a genuine cofactor in the single-enzyme reaction, functions in the luciferase-coupled reaction as a prebound substrate and is directly transferred to luciferase once it is reduced [Lei, B., and Tu, S.-C. (1998) *Biochemistry 37*, 14623−14629]. With the aim of better understanding the functions of FMN in the FRP holoenzyme, this study was undertaken to quantify and compare the thermodynamic properties of the binding of oxidized and reduced FMN by the FRP apoenzyme. By isothermal titration calorimetry (ITC) measurements in various buffers at pH 7.0 and 15−30 °C, the binding of FMN by apo-FRP was found to be noncooperative, exothermic, and primarily enthalpy driven. The binding free energy change (hence, the association constant) was nearly invariant over this temperature range. Significant conformational changes in FRP upon binding of FMN were indicated. Equilibrium bindings of reduced flavins by flavin-dependent proteins have rarely been studied. In this work, the thermodynamic properties of binding of reduced FMN by apo-FRP were found to closely resemble those of FMN binding under three sets of experimental conditions via ITC measurements and, in one case, fluorescence quenching. The kinetically deduced ping-pong mechanism of FRP is now supported by direct measurements of binding affinities of the oxidized and reduced FMN cofactors. These findings are also discussed in relation to the function of FRP as a reduced flavin donor in the FRP−luciferase couple.

In recent years, a growing number of "two-component" flavin-dependent monooxygenases have been identified (*1*). These two-component monooxygenaes have diverse functions, including bioluminescence (*2*, *3*), synthesis of antibiotics such as actinorhodin (*4*, *5*) and pristinamycin IIA (*6*, *7*), bioremediation of harmful chemicals such as the herbicide 2,4,5-trichlorophenoxyacetate (*8*, *9*), and desulfurization of fossil fuel (*10*−*12*). Each member of this enzyme family consists of a monofunctional monooxygenase and a flavin reductase (*1*). The former enzyme requires for its activity the supply of a reduced flavin from the latter enzyme.

The enzyme couple of luciferase and an NADPH-specific flavin reductase P (FRP)[1] from the luminous bacterium *Vibrio harveyi* are members of the flavin-dependent monooxygenase family. The FRP holoenzyme contains one bound FMN cofactor per monomer and catalyzes the reduction of a flavin substrate (such as FMN or riboflavin) by NADPH (eq 1), whereas luciferase catalyzes the oxidation of $FMNH_2$ and an aliphatic aldehyde (R-CHO) by oxygen to form FMN, carboxylic acid (R-COOH), water, and light (eq 2).

$$\text{FRP: } \mathrm{NADPH + H^+ + FMN \rightarrow NADP^+ + FMNH_2} \quad (1)$$

$$\text{luciferase: } \mathrm{FMNH_2 + R\text{-}CHO + O_2 \rightarrow FMN + R\text{-}COOH + H_2O + light} \quad (2)$$

This enzyme couple has been subjected to extensive studies with respect to their structures and mechanisms (*1*−*3*), the formation of the enzyme complex in vitro and in vivo (*13*, *14*), and direct channeling of reduced flavin from FRP to luciferase (*15*, *16*).

While the α and β subunits of bacterial luciferase remain tightly associated even at nanomolar concentrations (*17*), the FRP holoenzyme and apoenzyme readily participate in a monomer−dimer equilibrium with K_d values of 1.8 and 3.3 μM, respectively (*18*). Interestingly, luciferase forms a complex with the monomer of either the apoenzyme (K_d = 7 μM) or the holoenzyme (K_d = 11 μM) of FRP but not with FRP dimers (*13*). At optimal in vivo bioluminescence, *V. harveyi* cells contain ~170 μM luciferase and 3 μM FRP (*13*). Hence, the vast majority of FRP would be trapped in

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[1] Abbreviations: FRP, NADPH-specific flavin reductase; FMN and $FMNH_2$, oxidized and reduced riboflavin 5′-phosphate, respectively; Gnd·HCl, guanidine hydrochloride; CD, circular dichroism; ITC, isothermal titration calorimetry; ΔH_{obs}, observed enthalpy change; $\Delta H_{binding}$, intrinsic enthalpy of the protein−ligand binding interactions; ΔH_{ion}, ionization enthalpy of buffer; HEPES, 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid; PIPES, piperazine-1,4-bis(2-ethanesulfonic acid) 1,4-piperazinediethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid 4-morpholinepropanesulfonic acid.

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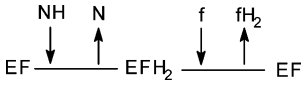
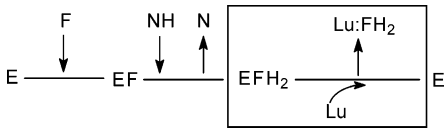
Scheme 1: FRP Single-Enzyme Reaction



Scheme 2: Coupled Reaction for the Direct Transfer of the $FMNH_2$ Cofactor



vivo in the form of a monomeric FRP−luciferase complex. Such a complex formation satisfies a prerequisite for direct channeling of $FMNH_2$ from FRP to luciferase. This complex formation also has an interesting mechanistic consequence. *V. harveyi* FRP, as a single enzyme, follows a ping-pong mechanism (*15*, *19*) as shown in Scheme 1, where E is the FRP apoenzyme, F and FH_2 are oxidized and reduced FMN cofactor, respectively, N and NH are $NADP^+$ and NADPH, respectively, and f and fH_2 are oxidized flavin substrate and reduced flavin product, respectively. The bound FMN cofactor co-isolated with FRP holoenzyme EF shuttles between F and FH_2 but remains bound to FRP during catalytic turnovers. However, FRP in the luciferase-coupled bioluminescence reaction follows a sequential mechanism (*15*) as depicted in Scheme 2 in which Lu is luciferase and all other abbreviations are the same as in Scheme 1. Once the bound FMN co-isolated with FRP holoenzyme is reduced by NADPH, it is directly transferred to luciferase for the subsequent bioluminescence reaction (not shown in Scheme 2). Such a direct $FMNH_2$ transfer (as highlighted with a box in Scheme 2) leaves FRP in the apoenzyme form, which binds a free FMN to regenerate the FRP holoenzyme. When viewed with the FRP apoenzyme as the starting point, a sequential mechanism for FRP is evident. In the coupled reaction, the FMN co-isolated with the FRP holoenzyme actually functions as a prebound flavin substrate (Scheme 2) contrary to the role as a genuine cofactor for FRP in the single-enzyme reaction (Scheme 1). To our knowledge, the switch of the functional role of an enzyme-bound flavin from a cofactor to a prebound substrate upon association with a functionally linked enzyme is unprecedented among known flavoproteins.

For all known flavoproteins, equilibrium bindings of reduced flavins have rarely been characterized. The nature of FMN and $FMNH_2$ binding by FRP is critical to our understanding of the catalysis by FRP in its single-enzyme and luciferase-coupled reactions. This work was undertaken to determine the thermodynamic properties of binding of FMN and $FMNH_2$ by apo-FRP. Findings are discussed in relation to the functions of FRP as a single enzyme and as an $FMNH_2$ donor for luciferase within this enzyme couple.

MATERIALS AND METHODS

General Materials. All chemicals were analytical grade and were used without further purification. Gnd·HCl was purchased from Promega. Urea was from EM Science. FMN and buffer reagents were from Sigma. The DC Protein Assay Reagent Package and bovine serum albumin were from Bio-Rad. Unless stated otherwise, 50 mM phosphate (P_i) buffer (pH 7.0) with molar fractions of 0.39 sodium monobase and 0.61 potassium dibase in deionized water was used as the standard buffer.

Expression and Purification of FRP. The expression of the cloned *V. harveyi* FRP holoenzyme and the purification to >95% purity were as described previously (*20*). The FRP apoenzyme was prepared from the purified holoenzyme with a Gnd·HCl/urea denaturation column and renatured by dilution into phosphate buffer following previously published procedures (*18*). Protein concentrations were determined by the method of Lowry (*21*) with bovine serum albumin as a standard.

Enzyme Assay. FRP holoenzyme activities were measured at 23 °C by monitoring the decrease in A_{340} associated with the oxidation of NADPH by using a Varian Cary 50 Bio UV−vis spectrophotometer. Details of the assay conditions were described previously (*15*).

Isothermal Titration Calorimetry. Binding of FMN by the FRP apoenzyme was assessed via ITC measurements using the VP-ITC isothermal titration calorimeter from Microcal, LLC (Northampton, MA). Prior to the ITC measurements, water, buffer, FRP apoenzyme, and FMN solutions were degassed for 10 min with gentle stirring under vacuum and carefully loaded into the cells and syringe to prevent the formation of bubbles. The reference cell was filled with water. The FRP apoenzyme was placed in the sample cell (1.45 mL) at a concentration of 3 μM, and 10 μL aliquots of FMN at 45 μM were stepwise injected into the sample cell with 300 s between two adjacent injections. The exposure of the FMN solution to light was kept to a minimum during the experiment to avoid photochemical reduction. During the titration, the reaction mixture was continuously stirred at 290 rpm. For each step of the titration, the binding heat was determined as the difference between the heat change generated after the injection of FMN into the apoenzyme solution and the corresponding background heat of titration, which was obtained by injecting FMN into the sample cell filled with an appropriate buffer solution under identical experimental conditions. Saturation of FRP by FMN binding was indicated when the observed heat change generated by any additional titration of FMN into the enzyme sample solution became identical to the background heat of titration. Thermodynamic parameters for the binding were determined from ITC results using Microcal ORIGIN version 7.0 (OriginLab Co., Northampton, MA) with the "ITC custom" add-on installed. The sensitivity of the binding isotherms is determined by a unitless parameter *c*, which is the product of the molar concentration of the macromolecule in the sample cell (in molar) and the binding constant (in inverse molar). Values of *c* between 5 and 500 are considered to be necessary for an accurate determination of association constants by ITC (*22*, *23*). Results of our ITC measurements were obtained under conditions with *c* values ranging from 10 to 200.

ITC measurements were carried out in various buffers (all at 50 mM), which are listed below along with their ionization enthalpies at 25 °C: MOPS (5.22 kcal/mol), phosphate (1.22 kcal/mol), HEPES (5.02 kcal/mol), and PIPES (2.74 kcal/mol) (*24*, *25*). Titrations were also performed at 15.0, 20.0, 25.0, and 30.0 °C in phosphate buffer.

For titration of the FRP apoenzyme by $FMNH_2$, apoenzyme and FMN solutions were made anaerobic by repeated

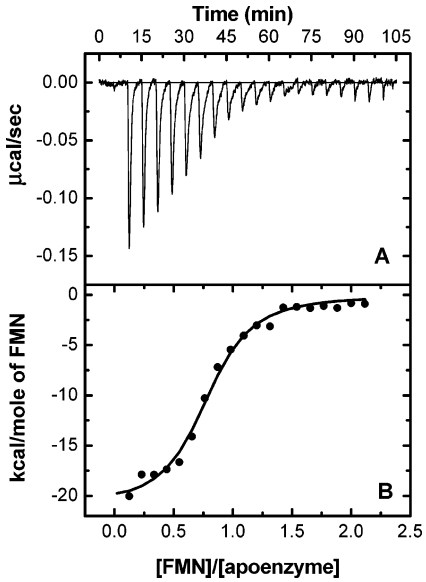


FIGURE 1: ITC measurements of binding of FMN by the FRP apoenzyme in 50 mM phosphate (pH 7.0) at 25 °C. Panel A shows the raw data, generated by titration of 3 μM FRP apoenzyme by 20 × 10 μL injections of 45 μM FMN. The area under each peak was integrated and plotted against the molar ratio of FMN to apoenzyme in panel B.

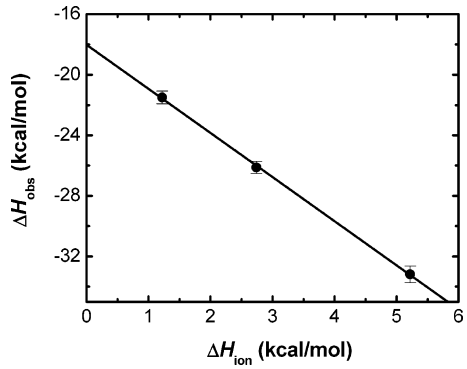


FIGURE 2: Observed enthalpy changes (ΔH_{obs}) of binding of FMN to apo-FRP as a function of buffer ionization enthalpy changes (ΔH_{ion}) at pH 7.0 and 25 °C. The slope (Δn, numbers of protons transferred per mole of apoenzyme monomer) and the abscissa intercept ($\Delta H_{binding}$, intrinsic binding enthalpy) of the linear regression of the data equaled −2.9 ± 0.1 and −18.0 ± 0.2 kcal/mol, respectively.

evacuations and saturations with nitrogen gas in airtight vessels. $FMNH_2$ was obtained by long-wavelength UV irradiation of the oxygen-free FMN solution (in a desired buffer containing 10 mM EDTA). The ITC apparatus was put in a glovebox which was constantly flushed with nitrogen gas prior to and during the experiment. $FMNH_2$ titrations were carried out following procedures otherwise identical to those described above for the FMN titrations.

Fluorescence Measurements of $FMNH_2$ Binding. The binding of $FMNH_2$ by the FRP apoenzyme was also assessed by protein fluorescence quenching measurements carried out at 23 °C in standard P_i buffer using a Varian Cary Eclipse fluorescence spectrophotometer. A fixed level of FRP apoenzyme and various concentrations of FMN, each in 1 mL of 50 mM phosphate buffer (pH 7.0) containing 10 mM EDTA, were placed in the cuvette and side arm, respectively, of an airtight glass tonometer (*26*). Repeated evacuations and equilibrations with nitrogen gas were performed over a 4 h period to remove oxygen from the samples. Reduction of FMN was achieved by photochemical reduction under long-wavelength UV irradiation. The $FMNH_2$ solution was then tilted into and mixed with the FRP apoenzyme solution. Fluorescence quenching of FRP upon $FMNH_2$ binding was monitored by setting the excitation at 280 nm and detecting the fluorescence emission at 330 nm. The reciprocal of Δfluorescence was plotted against the reciprocal of $FMNH_2$ concentration, where Δfluorescence is the fluorescence intensity of the apoenzyme in the absence of $FMNH_2$ minus that of the enzyme at a given $FMNH_2$ concentration.

RESULTS

ITC Measurements of Binding of FMN by Apo-FRP. The binding of FMN by apo-FRP was first assessed by ITC in 50 mM P_i (pH 7.0) at 25 °C and was shown to be an exothermic process (Figure 1A). The corresponding binding isotherm shown in Figure 1B can be best fitted as a noncooperative binding of FMN with an association constant (K_a) and an observed enthalpy change (ΔH_{obs}) of (6.50 ± 0.80) × 10^6 M^{-1} and −21.50 ± 0.42 kcal/mol, respectively. The K_a so determined corresponded to a K_d of 0.15 μM, which was consistent with a K_d of 0.20 μM measured previously in fluorescence experiments (*18*). After the ITC titration, the catalytic activity of the reconstituted FRP holoenzyme was recovered at a level similar to that of the native FRP holoenzyme (*27*).

It is known that the ΔH_{obs} determined directly from an ITC experiment contains contributions from both the intrinsic enthalpy of the protein−ligand binding interactions ($\Delta H_{binding}$) and the ionization enthalpy of the buffer species (ΔH_{ion}) (*28*−*30*). If the binding processes change the protonation states of free or complexed macromolecule and/or ligand, proton transfer between the binding complex and the buffer solvent occurs. As a consequence, ΔH_{obs} can be represented by eq 3:

$$\Delta H_{obs} = \Delta H_{binding} + \Delta n \Delta H_{ion} \quad (3)$$

where Δn is the number of protons that are released ($\Delta n > 0$) or taken up ($\Delta n < 0$) by the buffer medium upon binding (*28*−*30*). To determine $\Delta H_{binding}$ and Δn for binding of FMN by apo-FRP at pH 7.0 and 25 °C, ITC experiments in 50 mM P_i as shown in Figure 1 were repeated using 50 mM PIPES and MOPS buffers. The detected ΔH_{obs} values are plotted against the known ΔH_{obs} values for these three buffers (*24*). From the intercept on the abscissa and the slope of such a plot (Figure 2), values of $\Delta H_{binding}$ and Δn were determined to be −18.1 ± 0.2 kcal/mol and −2.9 ± 0.1, respectively.

Effects of Temperature on FMN Binding. A series of ITC experiments in 50 mM P_i (pH 7.0) were carried out at 15, 20, 25, and 30 °C to determine the $\Delta H_{binding}$ and K_a values for binding of FMN to apo-FRP. First, ΔH_{obs} results were analyzed on the basis of eq 3 in calculating $\Delta H_{binding}$. The temperature dependence of P_i buffer ΔH_{ion} has been determined previously (*24*), allowing the calculation of ΔH_{ion} values for P_i buffer in the range of 15−30 °C. On the basis of the independence of Δn from temperature previously reported for other enzymes at similar temperatures (*28*, *31*), the Δn of −2.9 determined at 25 °C for binding of FMN to

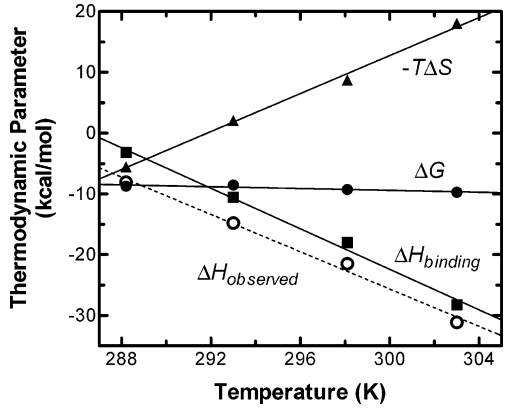


FIGURE 3: Thermodynamic parameters for the binding of FMN to FRP apoenzyme at pH 7.0 in the temperature range of 15−30 °C. The various buffers are described in the text. The ΔC_p was determined from the slope of the linear regression of the plot of $\Delta H_{binding}$ as a function of absolute temperature.

apo-FRP was assumed to remain unchanged in the range of 15−30 °C. Over this temperature range, the experimentally determined ΔH_{obs} and the calculated $\Delta H_{binding}$ are shown in Figure 3. Also shown are values of ΔG (determined as $\Delta G = -\ln K_a$) and $-T\Delta S$ (determined as $\Delta G = \Delta H_{binding} - T\Delta S$). The heat capacity ΔC_p for binding of FMN to apo-FRP was determined to be -1670 ± 100 cal mol^{-1} K^{-1} as the slope of the plot of $\Delta H_{binding}$ versus absolute temperature.

Circular Dichroism. CD spectra of FRP were recorded in 50 mM P_i (pH 7.0) at 23 °C using an OLIS DSM 1000 CD spectrophotometer. The native FRP holoenzyme and an apo-FRP sample with 92% of the FMN cofactor removed, each at 10 μM, exhibited no significant differences in their CD spectra in the range of 200−260 nm, indicating very little change of secondary structure upon cofactor binding.

Fluorescence Quenching Measurements of Binding of $FMNH_2$ by Apo-FRP. The binding of FMN to apo-FRP results in an 87% quenching of the protein fluorescence at 330 nm, and such a fluorescence quenching has been used as a signal for the determination of a K_d of 0.2 μM for FMN binding at 23 °C (*18*). In this work, a similar 88% quenching of the protein fluorescence at 330 nm was observed upon binding of $FMNH_2$ by apo-FRP (Figure 4A) at 23 °C in 50 mM P_i (pH 7.0). In a separate experiment, a limiting amount of apo-FRP (50 nM) was titrated with varying concentrations of $FMNH_2$. The reciprocal of Δfluorescence was plotted against the reciprocal of $FMNH_2$ concentration, where Δfluorescence is the fluorescence intensity of the apoenzyme in the absence of flavin minus that of the enzyme at a given $FMNH_2$ concentration. A dissociation constant for $FMNH_2$ binding at pH 7.0 and 23 °C was calculated to be 0.17 μM as the negative reciprocal of the intercept on the abscissa (Figure 4B).

ITC Measurements of Binding of $FMNH_2$ by Apo-FRP. The binding of $FMNH_2$ by apo-FRP was assessed by ITC measurements under anaerobic conditions in experiments otherwise identical to that described for Figure 1. In 50 mM P_i (pH 7.0) at 25 °C, $FMNH_2$ binding was also shown to be an exothermic process (Figure 5A). The corresponding binding isotherm (Figure 5B) gave rise to values of -21.8 ± 0.9 kcal/mol, $(6.9 \pm 1.2) \times 10^6$ M^{-1}, and -9.3 kcal/mol for ΔH_{obs}, association constant (K_a), and ΔG, respectively. The corresponding K_d is 0.15 μM, consistent with a K_d value

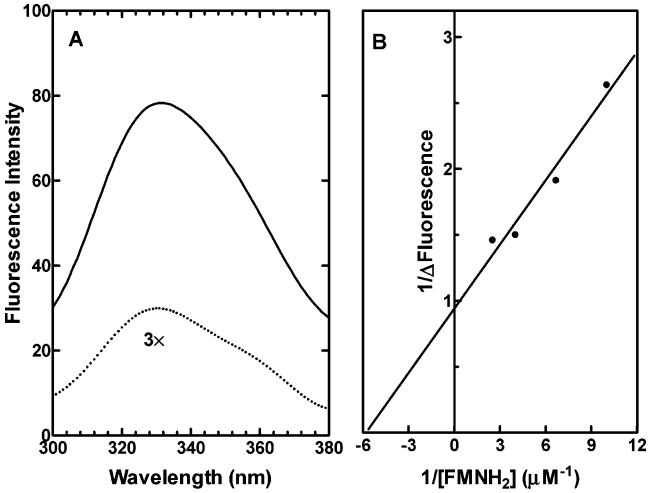


FIGURE 4: Detection of $FMNH_2$ binding by FRP fluorescence quenching. (A) Fluorescence spectra of the FRP apoenzyme, at a final concentration of 1 μM, without (—) and with 10 μM $FMNH_2$ (•••; intensity multiplied by 3 for presentation). All measurements were carried out in 50 mM phosphate and 10 mM EDTA (pH 7.0) at 23 °C using 280 nm excitation. The $FMNH_2$-containing sample was incubated for 10 min prior to spectral measurement. (B) The FRP apoenzyme at a final concentration of 50 nM was titrated with $FMNH_2$. The enzyme sample was incubated for 5 min after each addition of $FMNH_2$, and then fluorescence intensities at 330 nm were measured with 280 nm excitation. ΔFluorescence is defined as the difference in the emission intensity of the apoenzyme and that after the addition of a given amount of reduced flavin.

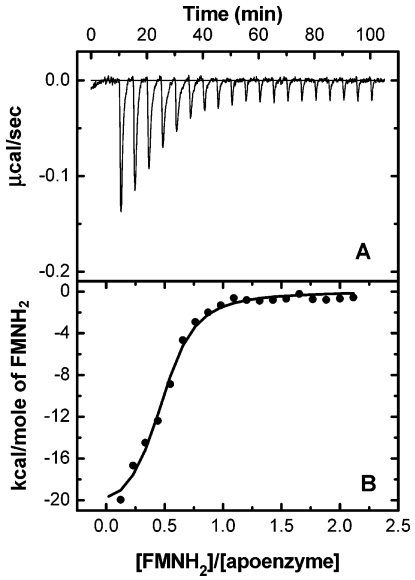


FIGURE 5: ITC measurements of binding of $FMNH_2$ by the FRP apoenzyme in 50 mM phosphate (pH 7.0) at 25 °C. Panel A shows the raw data, generated by titration of 3 μM FRP apoenzyme by 20 × 10 μL injections of 45 μM $FMNH_2$. The area under each peak was integrated and plotted against the molar ratio of $FMNH_2$ to apoenzyme in panel B.

of 0.20 μM measured in fluorescence experiments (*18*). The binding of $FMNH_2$ to apo-FRP was similarly assessed by ITC in 50 mM P_i (pH 7.0) at 30 °C and in 50 mM HEPES (pH 8.0) at 25 °C. Values of ΔH_{obs}, K_a, and ΔG so obtained are summarized in Table 1 in comparison with the corresponding values for FMN binding determined in experiments described above. Under these three sets of buffer and temperature conditions, the thermodynamic parameters for $FMNH_2$ binding closely resembled those for FMN binding.

Table 1: Comparison of the Thermodynamic Parameters for the Binding of FMN and $FMNH_2$ to the FRP Apoenzyme

buffer[a]/temperature	ligand	K_a ($\times 10^6$ M^{-1})	ΔH_{obs} (kcal/mol)	ΔG^b (kcal/mol)
P_i (pH 7.0)/25 °C	FMN	6.5 ± 0.8	-21.5 ± 0.4	-9.3
	$FMNH_2$	6.9 ± 1.2	-21.8 ± 0.9	-9.3
P_i (pH 7.0)/30 °C	FMN	9.2 ± 1.1	-31.1 ± 0.9	-9.7
	$FMNH_2$	9.2 ± 1.2	-31.8 ± 1.0	-9.6
HEPES (pH 8.0)/25 °C	FMN	35.8 ± 11.0	-23.2 ± 0.9	-10.3
	$FMNH_2$	34.7 ± 10.0	-22.3 ± 1.0	-10.2

[a] All buffers were at 50 mM. [b] Calculated from the relation $\Delta G = -RT \ln K_a$.

DISCUSSION

In this study, ITC measurements showed that the binding of FMN to apo-FRP in 50 mM P_i (pH 7.0) at 25 °C was a noncooperative process with a K_d of 0.15 μM (Figure 1). The total FRP concentration was at a constant level of 3 μM in these ITC titrations. Considering the K_d values of 1.8 and 3.3 μM for the dimer dissociation by the FRP holo- and apoenzyme, respectively (*18*), the enzyme sample used in this study should contain significant amounts of FRP monomers and dimers during the titrations. The monophasic binding isotherm shown in Figure 1B for a mixture of monomeric and dimeric FRP and the close correlation of the observed K_d of 0.15 μM for such a sample with the K_d of 0.2 μM for the binding of FMN to monomeric FRP (*18*) indicated that the FMN binding affinity of the dissociated FRP monomer was similar to that of the FRP monomer in the dimeric FRP. Monophasic binding isotherms were also observed for all other ITC measurements of FMN and $FMNH_2$ binding under a variety of conditions as described in this report. Considering that usual experimental conditions for kinetic and mechanistic studies often involve the coexistence of FRP monomers and dimers, the findings that both forms of FRP bind FMN and $FMNH_2$ similarly are important to the interpretation of experimental results.

The temperature dependences of FMN binding with respect to ΔG, $\Delta H_{binding}$ (obtained from ΔH_{obs} after corrections for Δn and ΔH_{ion}), and $T\Delta S$ are shown in Figure 3. Both $\Delta H_{binding}$ and $T\Delta S$ were highly dependent on temperature, whereas ΔG was almost invariant in the range of 15−30 °C due to an enthalpy−entropy compensation. Such an enthalpy−entropy compensation is often found in ligand−protein interactions (*28*, *32*−*35*). $\Delta H_{binding}$ had a negative value of −3.2 kcal/mol at 15 °C and became gradually more negative as the temperature increased to 30 °C. ΔS was found to switch from positive values at <19 °C to negative values at >19 °C. At 19 °C, $\Delta G = \Delta H_{binding}$. Hence, $\Delta H_{binding}$ is the sole driving force for FMN binding at this temperature. Under our experimental conditions, both $\Delta H_{binding}$ and ΔS contributed to the FMN binding at <19 °C, whereas FMN binding was driven by $\Delta H_{binding}$ but countered by ΔS at >19 °C. The large negative $\Delta H_{binding}$ and the negative ΔS (at >19 °C) indicate a significant contribution of hydrogen bonding and van der Waals interactions (*30*, *36*) to FMN binding, consistent with the nature of FMN binding by FRP revealed in the X-ray crystal structure (*37*). The heat capacity ΔC_p calculated from the slope of the regression line of $\Delta H_{binding}$ versus temperature in Figure 3 was -1670 ± 100 cal mol^{-1} K^{-1}. A negative ΔC_p value was normal in protein−ligand binding studies, usually indicating that an additional surface area was buried upon binding (*30*, *33*). Therefore, it is highly likely that FMN binding resulted in an increase in the buried surface area of FRP. However, we found that the CD spectrum of apo-FRP was essentially the same as that for the native holoenzyme. The secondary structures of the FRP apoenzyme were apparently not affected to any significant extent by FMN cofactor binding.

Experimentally determined values of ΔC_p and $\Delta H_{binding}$ could be used for a more detailed analysis of the conformational change upon binding of the ligand on the basis of the following relationships (*38*−*42*):

$$\Delta C_p = \Delta c_{ap}\Delta ASA_{ap} + \Delta c_{pol}\Delta ASA_{pol} \quad (4)$$

$$\Delta H_{binding}(60\ °C) = \Delta h_{ap}\Delta ASA_{ap} + \Delta h_{pol}\Delta ASA_{pol} \quad (5)$$

ΔASA_{ap} and ΔASA_{pol} are the changes in solvent accessible apolar and polar surface areas, respectively (in square angstroms), Δc_{ap} and Δc_{pol} are heat capacity changes per square angstrom due to apolar and polar surface area changes, respectively, Δh_{ap} and Δh_{pol} are the enthalpy contributions per square angstrom by changes in apolar and polar surfaces, respectively, and the enthalpy change at 60 °C can be determined by the relation $\Delta H_{binding}(60\ °C) = \Delta H_{binding} + \Delta C_p$ (60−25 °C). Using values of 0.45, −0.26, −8.43, and 31.3 for Δc_{ap}, Δc_{pol}, Δh_{ap}, and Δh_{pol}, respectively (*40*, *41*), ΔASA_{ap} and ΔASA_{pol} were calculated to be −6060 and −4060 Å^2, respectively, for the increased burial surface areas resulting from binding. However, it should be noted that somewhat different values can be assigned to parameters ΔASA_{ap}, ΔASA_{pol}, Δh_{ap}, and Δh_{pol} following different programs (*30*). Moreover, unrealistically large changes in solvent accessible surface areas upon ligand binding calculated from ΔC_p and ΔH have been noted before. In the case of binding of Na^+ to thrombin, the observed very high heat capacity change could be related to ordering of water molecules concomitant to changes of protein accessible areas (*43*). For formation of the apoflavodoxin−FMN complex, the value of accessible surface area calculated from crystallized structures was ~1 order of magnitude lower than the value calculated from the thermodynamic parameters (*42*). Therefore, the values of ΔASA_{ap} and ΔASA_{pol} calculated on the basis of the results of this work should be viewed, at the present, as an indication of significant conformational changes in terms of burial areas rather than accurate measurements of accessible surface area changes.

One major aim of this work is to compare FMN with $FMNH_2$ in the thermodynamic properties of their binding by apo-FRP. In the fluorimetric studies, the effects of $FMNH_2$ binding on the protein fluorescence spectrum and intensity of FRP (Figure 4A) were essentially identical to those of FMN binding (*18*). The K_d of 0.17 μM for $FMNH_2$ binding at pH 7.0 and 23 °C determined from results in Figure 4B also correlated well with the K_d of 0.2 μM for FMN binding (*18*). A more detailed comparison of FMN and $FMNH_2$ binding was conducted by additional ITC studies. In 50 mM P_i (pH 7.0) at 25 °C, not only K_a (and hence ΔG) but also ΔH_{obs} for $FMNH_2$ binding is essentially identical to those of FMN binding (Figure 5 and Table 1). The same similarities between FMN and $FMNH_2$ binding were also shown in terms of K_a, ΔG, and ΔH_{obs} in P_i buffer (pH 7.0) at 30 °C

and in Hepes at pH 8.0 and 25 °C (Table 1). Among the 16 hydrogen bonds between FMN and FRP, the crystal structure of FRP has shown that only the hydrogen bond between flavin N5 and the FRP Gly130 amide hydrogen could be lost as a direct consequence of flavin reduction (*37*). This structural information is fully consistent with our findings of the similarities between FMN and $FMNH_2$ with respect to their thermodynamic properties of binding. The similar binding properties observed for FMN and $FMNH_2$ could possibly be a consequence of reoxidation of $FMNH_2$ during our anaerobic fluorimetric and ITC titrations. This possibility was ruled out for the anaerobic fluorimetric titrations on the basis of the fact that no fluorescence at 535 nm for oxidized FMN was detected during the entire course of titration. For ITC titrations, the entire calorimeter and all O_2-free reagents and titration syringes were placed in a glovebox, which was flushed with nitrogen gas thoroughly prior to and constantly during the ITC experiments. The ITC reaction cell and the titration syringe were thoroughly washed with O_2-free buffer prior to the addition of the enzyme sample and $FMNH_2$ titration. The same general treatment has been successfully applied in our hands to anaerobic stopped-flow experiments involving reduced flavins (*44*). Moreover, the K_d for $FMNH_2$ binding determined by the fluorimetric titrations in 50 mM P_i (pH 7.0) at 23 °C was essentially the same as that obtained from ITC titrations in the same buffer at 25 °C. Furthermore, ITC titrations using FMN and $FMNH_2$ all exhibited monophasic isotherms. Taken together, we believe that the $FMNH_2$ sample remained reduced during our ITC experiments and FMN and $FMNH_2$ were similar in their binding to FRP.

Steady-state kinetic studies support a ping-pong mechanism (Scheme 1) for the FRP single-enzyme reaction (*15*, *19*). This mechanism requires that the FMN cofactor, oxidized or reduced, remains bound during catalysis. Accordingly, FRP cannot tolerate a weak binding for either the oxidized or the reduced FMN cofactor. Indeed, our fluorimetric and ITC results showed that apo-FRP bound the FMN and $FMNH_2$ cofactor equally well with a low dissociation constant of ~0.2 μM at pH 7.0 and 25 °C. The validity of the kinetically deduced ping-pong mechanism of FRP is now supported by direct measurements of equilibrium bindings of FMN and $FMNH_2$. To our knowledge, no other ping-pong type flavoenzymes have been examined by such a direct comparison of the bindings of oxidized and reduced flavin cofactors.

Bacterial luciferase relies on the supply of $FMNH_2$ from flavin reductase for its bioluminescence activity. For the *V. harveyi* FRP−luciferase couple, our previous kinetic studies and the use of 2-thioFMN as a mechanistic probe both indicate that the reduced flavin cofactor, rather than the product, of FRP is directly transferred to luciferase in their coupled reaction (*15*) as shown in Scheme 2. For such a direct transfer of $FMNH_2$, FRP must form a complex with luciferase. We have shown that monomeric FRP indeed forms a functionally active complex with luciferase in vitro and in vivo (*13*, *14*). Scheme 2 is further validated in this study on the basis of the binding affinities for FMN and $FMNH_2$ of luciferase and FRP. Luciferase binds FMN with a K_d of ~0.12 mM (*45*, *46*), much weaker than the affinity of FRP for FMN binding ($K_d \sim 0.2$ μM) determined previously (*18*) and in this work. Therefore, direct transfer of FMN from FRP to luciferase would not be a favorable process. In contrast, luciferase binds $FMNH_2$ with a K_d of 0.8 μM (*47*) in comparison with a K_d of 0.15−0.17 μM for the binding of $FMNH_2$ by FRP determined in this work. Although luciferase does not bind $FMNH_2$ any tighter than FRP, their comparable binding affinities for $FMNH_2$ should allow an effective partition of $FMNH_2$ between luciferase and FRP. Moreover, due to its weak binding of FMN, luciferase is poised to release the FMN formed in the bioluminescence reaction to allow the binding of FMN by FRP, either by diffusion or by channeling, enabling the turnover of the steps shown in Scheme 2. It should, however, be noted that the above rationalization is based on the assumption that binding affinities of luciferase and FRP for FMN and $FMNH_2$ are not markedly affected by the formation of the complex of these two enzymes. The validity of this assumption cannot be evaluated by the results of this work and should be investigated further.

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