Thermodynamics of Ferredoxin Binding to Ferredoxin:NADP$^+$ Reductase and the Role of Water at the Complex Interface†

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ABSTRACT: The association of ferredoxin with ferredoxin:NADP$^+$ reductase (both proteins from spinach chloroplasts) was characterized by isothermal titration calorimetry and fluorescence quenching titration. The formation of the complex is mainly driven by a positive entropy change (ΔS = 125 ± 8 J mol^{-1} K^{-1}). The calorimetric enthalpy of binding is small between 10 and 37 °C and either negative or positive, with an inversion temperature near 25 °C. The pH dependence of the association constant [Batie, C. J., & Kamin, H. (1981) *J. Biol. Chem. 256,* 7756−7763] was shown to correlate with the uptake of a single proton by a group exhibiting a heat of protonation of −26 kJ mol^{-1}. This value agrees with the protonation of an imidazole group. Possible residues to become protonated in the complex are His-19 or His-90 of ferredoxin:NADP$^+$ reductase. The temperature dependence of the free energy of binding, ΔG, is weak because of the enthalpy−entropy compensation caused by a heat capacity change, ΔC_p, of −680 ± 44 J mol^{-1} K^{-1}. The favorable binding entropy and the negative ΔC_p indicate a large contribution to binding from hydrophobic effects, which seem to originate from dehydration of the protein−protein interface. Dehydration was demonstrated by osmotic stress experiments in which the association constant was found to increase by 2−4-fold in the presence of 52% (w/w) glycerol. The increase in the association constant with osmotic pressure points to the release of several water molecules from the complex interface.

Many biological oxidation−reduction reactions depend on the specific association of proteins containing redox-active prosthetic groups. The recognition sites on the surfaces of complementary electron transfer partners have been studied in many laboratories, and residues contributing to the formation and specificity of several complexes have been identified (Smith et al., 1977; Rieder & Bosshard, 1978; Ferguson-Miller et al., 1978; Weber & Tollin, 1985; Rush et al., 1988; Farver & Pecht, 1991; Medina et al., 1992; De Pascalis et al., 1993). The high-resolution X-ray structure of at least two electron transfer complexes has been solved (Pelletier & Kraut, 1992; Chen et al., 1994). Ionic interactions contribute to the stability of most electron transfer complexes, and the complex interface seems to coincide with surface areas of oppositely charged potentials (Margoliash & Bosshard, 1983; Matthew et al., 1983; Mauk et al., 1986; Northrup et al., 1987; Roberts et al., 1991; De Pascalis et al., 1993). This common property of electron transfer complexes raises important questions about the thermodynamics of the association of electron transfer proteins, particularly about the enthalpic and entropic contributions to the free energy of binding and the role of water at the interface of the electrostatically stabilized complexes.

Among the relatively few protein−protein complexes for which thermodynamic data are available, there appears to be a group of complexes characterized by a large favorable enthalpy of binding that is partly offset by an unfavorable entropy term. Most antigen−antibody complexes studied so far belong to this group (Tello et al., 1993). There is a contrasting group of protein complexes that are stabilized by a large positive entropy of binding, partly compensated by an unfavorable enthalpic term. Examples are found among protease−inhibitor complexes (Bough & Trowbridge, 1972). Within the limited data set available, there are surprisingly few cases in which both the enthalpic and the entropic contributions are favorable [see Table 4 in Hibbits et al. (1994)].

Here we have analyzed the association of the [2Fe−2S] protein ferredoxin (Fd)[1] with ferredoxin:NADP$^+$ reductase (FNR) by isothermal titration calorimetry (ITC). FNR catalyzes the terminal step of the electron transport chain in chloroplast thylakoids, namely, the reduction of NADP$^+$ to NADPH by 2 equiv of reduced Fd [reviews by Knaff and Hirasawa (1991) and Zanetti and Aliverti (1991)]. Fd and FNR from spinach leaf chloroplasts form an electrostatically stabilized 1:1 complex. Formation of the complex can be followed from the change in the visible absorption spectrum of the proteins (Foust et al., 1969) or from the quenching of the tryptophan fluorescence of FNR (Davis, 1990). Fd contributes the negative charges and FNR the positive charges to the Fd:FNR complex, for which we have proposed a hypothetical binding model based on protein chemical studies and surface potential calculations (De Pascalis et al., 1993; Jelesarov et al., 1993). A related model was inferred from a comparison of the X-ray structures of FNR and Fd with that of phthalate dioxygenase reductase (Correll et al., 1993).

Foust et al. (1969) noted that the difference absorption spectrum characterizing the Fd:FNR complex is temperature insensitive, which led them to postulate that the formation

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[1] Abbreviations: eu, entropic units; Fd, ferredoxin; FNR, ferredoxin:NADP$^+$ reductase; ITC, isothermal titration calorimetry; Mops, 4-morpholinopropanesulfonic acid.

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of the complex is mainly driven by an increase in entropy. An entropic contribution to binding could arise from hydrophobic effects and electrostatic interactions (Chothia & Janin, 1975; Ross & Subramanian, 1981; Janin & Chothia, 1990). Close intermolecular contacts require the dehydration of at least some parts of the interacting surfaces. The entropic gain from the transfer of interfacial water into the bulk solvent is a source of binding energy. Some authors regard this effect as the main factor to stabilize protein–protein complexes (Chothia & Janin, 1975; Miller et al., 1987). However, it is a matter of controversy whether exclusion of water from the complex interface promotes or opposes binding. van der Waals interactions and hydrogen bonds in a low dielectric medium at the complex interface may be entropically unfavorable, but they make a favorable enthalpic contribution to the stability of the complex (Bhat et al., 1994).

We found that the association of Fd with FNR is dominated by a favorable entropy change. A positive ΔS most likely originates from the removal of several water molecules from the complex interface. This was concluded from osmotic stress experiments in which the association constant, K_A, increased when the titration was conducted in water/glycerol mixtures. Glycerol reduces the activity of bulk water and forces the release of bound water from the complex interface (Rand et al., 1993). The calorimetric enthalpy change, which is close to 0 in the region 10–37 °C, could be separated into a favorable negative $\Delta H_{protonation}$ and an unfavorable positive $\Delta H'$ of unknown origin. $\Delta H_{protonation}$ is caused by the uptake of a single proton, most likely by a histidine residue of FNR.

MATERIALS AND METHODS

Materials. FNR was purified from spinach leaves (Shin & Oshino, 1978) and stored at −80 °C in 50 mM Tris buffer (pH 8.0). The native 35 kDa form of FNR may be proteolytically degraded during purification and storage (Shin et al., 1990). The material we used was free of the truncated form as tested by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Purified FNR had an absorbance ratio A_{456}/A_{280} of 0.125–0.130. The procedure of Tagawa and Arnon (1968) was followed to isolate Fd. Unless otherwise noted, isoform I of Fd was used in all experiments. Isoforms I and II were separated and purified according to Ohmori et al. (1989) and stored at −80 °C in 0.2 M sodium phosphate buffer (pH 7.0). The absorbance ratio A_{420}/A_{276} of Fd isoforms I and II was 0.45–0.46. Concentrations of FNR and Fd were determined using absorbance coefficients of 9680 M^{-1} cm^{-1} at 420 nm for Fd (Tagawa & Arnon, 1968) and 10 740 M^{-1} cm^{-1} at 460 nm for FNR (Forti et al., 1970). Anhydrous glycerol (Fluka) was stored under argon. Glycerol solutions were prepared gravimetrically immediately before use.

Isothermal Titration Calorimetry (ITC). All calorimetric experiments were performed with an OMEGA titration calorimeter (MicroCal, Inc., Northampton, MA). The instrument has been described by Wiseman et al. (1989). The calorimeter was calibrated with electrically generated heat pulses as recommended by the manufacturer. To improve baseline stability, the temperature of the system was kept about 5 °C below the temperature of the actual experiment with the help of a circulating water bath. Temperature equilibration was allowed for 10–12 h. All solutions were thoroughly degassed by stirring under vacuum before use. Protein samples were prepared in buffer of the same batch to minimize artifacts due to minor differences in buffer composition. The reaction cell contained 1.33 mL of 10 μM FNR in buffer; the reference cell contained 0.02% sodium azide in water. The injection syringe was filled with 200 μM Fd in buffer and was rotated at 350 rpm during equilibration and experiment. Injections were started after equilibration to baseline stability (rms noise <5 ncal s^{-1}, baseline drift <10 ncal min^{-1}).

A titration experiment consisted of 25 injections, each of 5 μL volume and 10 s duration, with a 4.5 min interval between injections. The titration data were corrected for the small heat changes observed in control titrations of buffer into buffer and Fd into buffer. Data analysis was carried out with the software provided with the instrument (Wiseman et al., 1989). The *c*-value, defined as the concentration of FNR multiplied by the binding constant K_A, was 20–300. *c*-values of this magnitude guarantee a reliable estimate of K_A (Wiseman et al., 1989). The total apparent heat of binding, $\Delta H_{observed}$, and K_A were obtained by nonlinear least-squares fitting of the data to a 1:1 binding model utilizing the Marquardt algorithm. The software also provided an estimate of the stoichiometry, *n*, which can serve as an internal check of accuracy. Values of *n* for all experiments deviated from the expected value of 1 by no more than 5%, indicating that the protein concentrations were determined precisely and the binding model was correct. Analysis of the content of the reaction cell after titration confirmed the presence of an Fd:FNR complex of stoichiometry 1:1 and an excess of Fd as well as the absence of free FNR. The analysis was performed by gel chromatography on Superdex-75 (Pharmacia FPLC system) as described previously (Jelesarov et al., 1993).

The following buffers were used: 50 mM (ionic strength $\mu = 41$ mM) Tris-HCl (pH 7.5); 50 mM ($\mu = 35$ mM) Mops (pH 7.5); 25 mM ($\mu = 50$ mM) sodium phosphate (pH 7.5); 50 mM ($\mu = 18$ mM) sodium cacodylate (pH 6.0); 50 mM ($\mu = 40$ mM) glycine/NaOH (pH 9.2); 50 mM ($\mu = 26$ mM) sodium borate (pH 9.2). The pH value of a buffer was adjusted at the working temperature (10–37 °C). The enthalpies of ionization of these buffers are as follows: Tris, 47.35 kJ mol^{-1}; Mops, 22.11 kJ mol^{-1}; phosphate, 5.1 kJ mol^{-1}; cacodylate, −2.34 kJ mol^{-1}; glycine, 43 kJ mol^{-1}; borate, 13.8 kJ mol^{-1} (Morin & Freire, 1991; Christensen et al., 1976). We found no literature value for the enthalpy of ionization of Tris in glycerol/water mixtures. Therefore, 50 mM Tris was titrated with HCl by ITC in the presence of glycerol to obtain enthalpies of ionization at 27 °C of 51.41 kJ mol^{-1} for 26% glycerol and 55.59 kJ mol^{-1} for 52% glycerol. Enthalpies of ionization were constant within experimental error in the temperature range 10–37 °C. An increase of similar magnitude in the enthalpy of ionization was observed in ethylene glycol (Christensen et al., 1976).

Determination of Dissociation Constants by Fluorescence Quenching Titration. Fluorescence was measured with a Spex Fluorolog spectrofluorimeter (Spex Industries, Inc., Edison, NJ) connected to a circulating water bath to control the temperature measured in the cuvette within ±0.2 °C. FNR (1.5 μM in a total volume of 1.8 mL of 50 mM Tris buffer, pH 7.5) was titrated with 16 2 μL aliquots of 0.189 mM Fd in the same buffer. Fluorescence emission was measured

Table 1: Thermodynamic Parameters of Binding of Fd to FNR in 50 mM Tris-HCl Buffer (pH 7.5)[a]

T (K)	number of experiments[b]	$K_A \times 10^{-7}$ (L mol^{-1})	ΔG[c] (kJ mol^{-1})	ΔH[d] (kJ mol^{-1})	$T\Delta S$ (kJ mol^{-1})
283	3 cal	0.49 ± 0.12 (0.041)	−36.26 ± 0.64 (0.47)	4.35 ± 1.80 (1.71)	40.61 ± 2.44
292	1 cal	0.53 ± 0.27	−37.58 ± 1.75	8.27 ± 3.87 (−)	45.85 ± 5.62
300	5 cal	0.65 ± 0.19 (0.012)	−39.11 ± 0.88 (0.45)	−1.31 ± 1.35 (2.06)	37.80 ± 2.23
				1.03 ± 1.46[e]	
	3 cal[g]	0.51 ± 0.2 (0.011)[g]	−38.52 ± 1.2 (0.48)[g]	−5.95 ± 1.82 (1.80)[g]	32.57 ± 2.28[g]
	2 flu	0.42 ± 0.02 (0.014)	−38.04 ± 0.13 (0.08)		38.04 ± 1.5[f]
	2 flu[g]	0.37 ± 0.03 (0.011)[g]	−37.72 ± 0.21[g]		31.77 ± 0.4[g,h]
305	1 cal	0.75 ± 0.29	−40.14 ± 1.25	−8.56 ± 1.42	31.58 ± 2.67
310	4 cal	0.99 ± 0.47 (0.074)	−41.51 ± 1.67 (0.66)	−12.76 ± 1.33 (3.6)	28.75 ± 3.00

[a] Numbers are mean ± mean experimental error, followed by the standard deviation of the mean in parentheses. [b] cal, data obtained by ITC; flu, data obtained by fluorescence quenching titration. [c] Calculated from K_A. [d] Values after subtraction of the ionization enthalpy of Tris buffer (47.4 kJ mol^{-1}). [e] Value from the best fit of data to eq 3. [f] Calculated assuming $\Delta H = 0$ at 27 °C. [g] Values for Fd isoform II. [h] Calculated with $\Delta H = -5.95$ kJ mol^{-1} determined by ITC.

at 347 nm (excitation wavelength, 295 nm; integration time, 60 s). Using the law of mass action, the relative fluorescence change, $\Delta F/\Delta F_{max}$, for the equilibrium FNR + Fd ⇌ Fd:FNR can be expressed as a function of the concentration of free [Fd]:

$$\Delta F/\Delta F_{max} = [Fd]/([Fd] + K_d) \quad (1)$$

where $K_d = 1/K_A$ and [Fd] is the root of the equation $[Fd]^2 - [Fd](K_d + [FNR]_0 - [Fd]_0) - K_d[Fd]_0 = 0$. $[FNR]_0$ and $[Fd]_0$ are the total concentrations of FNR and Fd, respectively. K_d and ΔF_{max} were determined by a nonlinear least-squares fit of eq 1 to the data.

Analysis of the Data from Binding Experiments in the Presence of Glycerol. Water bound to a protein−protein interface may behave as though it were separated from the bulk solvent by an imaginary semipermeable membrane (Rand et al., 1993; Kornblatt et al., 1993). Decreasing the water activity W in the bulk solution by adding the osmotically active cosolvent glycerol should shift the equilibrium at the interface toward the less hydrated state. If the volume of osmotically labile water is ΔV_w, then osmotic work equal to $\pi\Delta V_w$ would appear as a contribution to the overall free energy ΔG of binding of Fd to FNR (Rand et al., 1993):

$$\Delta G = RT \ln K_d + \pi\Delta V_w = \text{constant}$$

where

$$\pi\Delta V_w = RT \ln W$$

If we determine the dissociation constant in buffer (K_d^o) and in buffer/glycerol mixtures (K_d^π), we obtain

$$RT \ln(K_d^\pi/K_d^o) = \Delta\pi\Delta V_w \quad (2)$$

where $\Delta\pi$ is the change in osmotic pressure produced by the addition of glycerol. K_d^o and K_d^π were determined by ITC and fluorescence quenching titration, and the data were plotted according to eq 2 to obtain ΔV_w. The osmotic pressure was calculated from

$$\pi\Delta V_{H_2O} = RT \ln X_{H_2O}$$

where ΔV_{H_2O} is the partial molal volume of water and X_{H_2O} is the mole fraction of water at the corresponding glycerol concentration [*Handbook of Chemistry and Physics*, 70th ed. (1989−1990) p D234, CRC Press, Boca Raton, FL].

RESULTS AND DISCUSSION

Microcalorimetric and Fluorimetric Determination of the Association Constant of the Fd:FNR Complex. ITC measures the calorimetric enthalpy change, ΔH, when Fd binds to FNR. Provided one can conduct the titration experiment in the appropriate range of protein concentrations, ITC also yields the association constant K_A and, thus, ΔG of the Fd:FNR complex (Wiseman et al., 1989). The entropy of binding, ΔS, can then be calculated from $\Delta G = \Delta H - T\Delta S$.

Affinity constants of the Fd:FNR complex were determined in 50 mM Tris buffer (pH 7.5) in the temperature range 10−37 °C by ITC and fluorescence quenching titration (Table 1). The mean value of K_A at 27 °C from ITC was (6.45 ± 1.9) × 10^6 M^{-1} (five measurements) and that from fluorescence quenching was (4.2 ± 0.3) × 10^6 M^{-1} (two measurements). This difference is small in view of the large experimental uncertainty in the ITC experiments. However, K_A values from fluorescence quenching were consistently lower at 10 °C also and when measured in the presence of glycerol (see the following and Table 2). Fd isoforms I and II, which differ at 26 of 97 sequence positions, bind to FNR with the same affinity (Table 1). K_A depends only weakly on temperature in the narrow range tested. The value from ITC increases by about 2-fold from 10 to 37 °C. Batie and Kamin (1981) obtained $K_A = 1 \times 10^6$ M^{-1} from difference absorption spectroscopy under the same buffer conditions used here and at ambient temperature.

Observed Calorimetric Enthalpy of Reaction Dependence on the Ionization Heat of the Buffer. Binding of Fd to FNR is pH dependent (Foust et al., 1969; Batie & Kamin, 1981). The dependence was interpreted by a single protonation event caused by a pK shift of a titratable group from about 6 (free) to about 8.2 (complex). If binding involves a change in the protonation state of FNR (or Fd), protons are exchanged with the buffered medium. To obtain the true binding enthalpy, $\Delta H_{binding}$, the observed heat of reaction, $\Delta H_{observed}$, must be corrected to account for the heat of ionization of the buffer, ΔH_{buffer}, according to

$$\Delta H_{observed} = \Delta H_{binding} + n_{H^+}\Delta H_{buffer} \quad (3)$$

where n_{H^+} is the number of protons released by the buffer. ITC experiments were performed in buffers of different heats of ionization, and the data were analyzed according to eq 3 (Figure 1). The slope of Figure 1 is 0.96 ± 0.03. Thus, a single proton is released by the buffer for each molecule of Fd bound to FNR. Extrapolation of the data in Figure 1 to zero buffer ionization heat yields $\Delta H_{binding} = 1.03 \pm 1.46$ kJ mol^{-1} at 27 °C. At the same temperature, a mean value of $\Delta H_{binding} = -1.31 \pm 1.35$ kJ mol^{-1} was obtained from five measurements in Tris buffer, after subtracting the heat of protonation of Tris of 47.4 kJ mol^{-1} (Table 1).

Table 2: Thermodynamic Parameters of Binding of Fd to FNR in 50 mM Tris-HCl Buffer (pH 7.5) and at Different Concentrations of Glycerol[a]

glycerol (wt %)	number of experiments[b]	T (°C)	$K_A \times 10^{-7}$ (L mol^{-1})	ΔG^c (kJ mol^{-1})	ΔH^d (kJ mol^{-1})
0	5 cal	27	0.65 ± 0.19 (0.12)	−39.11 ± 0.88 (0.45)	−1.31 ± 1.35 (2.06)
	2 flu		0.42 ± 0.02 (0.014)	−38.04 ± 0.13 (0.08)	
26	3 cal	27	1.50 ± 0.58 (0.39)	−41.21 ± 1.22 (0.61)	−19.60 ± 0.88 (0.93)
	1 flu		0.6 ± 0.03	−38.97 ± 0.11	
52	3 cal	27	2.59 ± 1.58 (0.91)	−42.58 ± 2.35 (0.37)	−31.08 ± 1.20 (2.19)
	5 flu		0.82 ± 0.09 (0.07)	−38.80 ± 0.3 (0.22)	
52	3 flu	9	0.53 ± 0.062 (0.07)	−36.30 ± 0.28 (0.3)	
52	3 flu	19	0.49 ± 0.087 (0.03)	−37.39 ± 0.47 (0.14)	
52	3 flu	40	1.00 ± 0.40 (0.06)	−41.94 ± 1.32 (0.15)	

[a] Numbers are mean ± mean experimental error, followed by the standard deviation of the mean in parentheses. [b] cal, data obtained by ITC; flu, data obtained by fluorescence quenching titration. [c] Calculated from K_A. [d] Values are the total heat measured in the ITC experiment after subtraction of the ionization enthalpy of Tris buffer, which was determined by ITC at the corresponding glycerol concentration and temperature (see Materials and Methods).

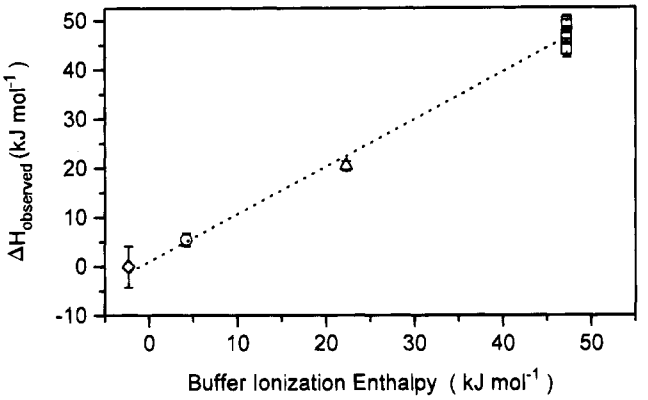

FIGURE 1: Observed enthalpy change for the binding of Fd to FNR at 27 °C as a function of the ionization enthalpy of the buffer. ITC was performed at pH 7.5 in Tris (□), Mops (△), and phosphate buffer (○) and at pH 6.0 in cacodylate buffer (◇). Dashed line: linear least-squares fit to eq 3. Slope = 0.96 ± 0.03; intercept at zero buffer ionization enthalpy = 1.03 ± 1.46 kJ mol^{-1}.

$\Delta H_{binding}$ can be thought to be composed of a term that does not depend on the protonation event, $\Delta H'$, and a term describing the contribution of the protonation of FNR (or Fd) to the enthalpy change:

$$\Delta H_{binding} = \Delta H' + n_{H^+}\Delta H_{protonation} \quad (4)$$

From eqs 3 and 4,

$$\Delta H_{observed} = \Delta H' + n_{H^+}(\Delta H_{protonation} + \Delta H_{buffer}) \quad (5)$$

Between pH 9 and 10, binding of Fd to FNR is pH independent (Batie & Kamin, 1981). Therefore, n_{H^+} of eq 5 becomes zero. Indeed, titration at pH 9.2 in borate buffer (ΔH_{buffer} = 13.8 kJ mol^{-1}) and in glycine buffer (ΔH_{buffer} = 43 kJ mol^{-1}) yielded the same apparent heats of reaction: $\Delta H_{observed}$ = 25.5 ± 0.5 kJ mol^{-1} (borate) and $\Delta H_{observed}$ = 26.5 ± 0.9 kJ mol^{-1} (glycine).[2] Thus $\Delta H'$ is 26 ± 0.9 kJ mol^{-1}, the mean of these two values. Assuming that $\Delta H'$ does not vary measurably in the pH range 7.5−9.2, we may use $\Delta H'$ = 26 ± 0.9 kJ mol^{-1} at pH 7.5. At this pH, n_{H^+} = 1 and $\Delta H_{binding}$ is near 0 (Table 1, Figure 1). Hence, it follows from eq 4 that $\Delta H'$ and $\Delta H_{protonation}$ almost cancel, and thus $\Delta H_{protonation}$ is about −26 kJ mol^{-1}. This is the net enthalpic contribution of the protonation of FNR (or Fd) at pH 7.5 and 27 °C.

Interestingly, $\Delta H_{binding}$ of Fd isoform II was slightly but significantly more negative than $\Delta H_{binding}$ of isoform I at 27 °C and pH 7.5. Since K_A was the same for both isoforms, $T\Delta S$ for isoform II was less positive (Table 1). This was the only difference between the two isoforms that we have observed.

Nature of the Group Responsible for Proton Uptake. The heats of protonation for normal carboxyl, ε-amino, α-amino, phenolic, and imidazole groups in globular proteins are 0, −43.9, −41.8, −26.3, and −26.3 kJ mol^{-1}, respectively (Shiao & Sturtevant, 1976). The values for His and Tyr coincide with a $\Delta H_{protonation}$ of −26 kJ mol^{-1}. Protonation of a tyrosine is very unlikely in view of a pK of 6 for the protonatable group in the free protein (Batie & Kamin, 1981). We have performed theoretical calculations of the pK values of side chains of FNR and of several Fd's and found no anomalously down-shifted pK values for tyrosines.[3]

What is the evidence for protonation of a residue on FNR? First, the same pH dependence was seen for the binding of FNR to Fd and to flavodoxin, two unrelated proteins (Batie & Kamin, 1981). Therefore, the protonated group is more likely to be on FNR. Second, Fd contains only a single His, which is peripheral to the putative binding site for FNR (De Pascalis et al., 1993). On the other hand, there are two His within the likely binding site of FNR for Fd. One is His-90, located in one of the two positive potential domains that contribute to the Fd binding site (De Pascalis et al., 1993). The other is His-19 adjacent to Lys-18, which is protected from chemical modification in the Fd:FNR complex (Jelesarov et al., 1993). Although the evidence for the protonation of a His of FNR is indirect, we believe it is definitely stronger than the evidence for protonation of the single His of Fd.

Heat Capacity Change ΔC_p and Temperature Dependence of the Thermodynamic Parameters. Within the experimental errors of ITC measurements, which were relatively large because of the small observed heats of reaction, $\Delta H_{binding}$ depends linearly on temperature in the range 10−37 °C. ΔC_p

[2] The UV and visible absorbance spectra of Fd and FNR did not change at pH 9.2 and 27 °C during the 2.5 h of the ITC experiment, an indication that protein structures were not significantly perturbed at pH 9.2. The known decrease in K_A at alkaline pH is primarily attributed to the change in protonation. The relative decrease we observed at pH 9.2 was of the same magnitude as that reported by Batie and Kamin (1981) (data not shown).

[3] Coordinates were from PDB entries 2FNR, 1FXI, 1FXA, and 1FXC; calculations were performed with program MEAD, version 0.2.2 (Bashford & Karplus, 1990). Atomic radii and partial atomic charges were assigned according to the CHARMM19 parameter set.

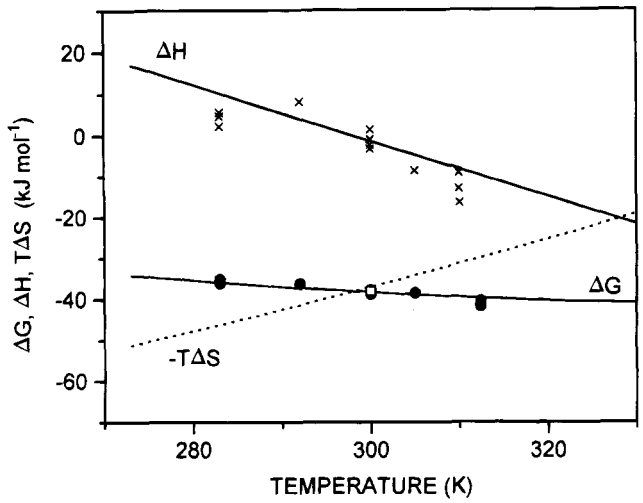

FIGURE 2: Thermodynamic parameters for binding of Fd to FNR as a function of temperature: (×) enthalpies of binding determined by isothermal titration calorimetry. These data were used to calculate ΔC_p. ○: ΔG calculated from K_A obtained by ITC. □: ΔG calculated from K_A obtained by fluorescence quenching at 27 °C.

$= -680 \pm 44$ J K^{-1} mol^{-1} was obtained from the slope of ΔH in Figure 2. If ΔC_p is temperature independent, enthalpy and entropy changes can be expressed as

$$\Delta H = \Delta H^* + \Delta C_p(T - T^*_h)$$
$$\Delta S = \Delta S^* + \Delta C_p \ln(T/T^*_s)$$

ΔH^* and ΔS^* are the enthalpy and entropy changes at the reference temperatures T^*_h and T^*_s, respectively. From these equations and the standard thermodynamic relationship $\Delta G = \Delta H - T\Delta S$ follows

$$\Delta G = \Delta H^* - T\Delta S^* + \Delta C_p[(T - T^*_h) - T \ln(T/T^*_s)]$$

Using $T^* = 300$ K (the temperature where the largest data set was obtained; Table 1), we get the temperature change of the thermodynamic parameters shown in Figure 2. ΔG changes little with temperature because of enthalpy−entropy compensation, as has been found for many protein−protein association reactions. The binding enthalpy varies from weakly positive to weakly negative and changes sign near 27 °C. The entropy term $T\Delta S$ is large in magnitude and positive in the physiological range of temperature. Only above 60 °C does $T\Delta S$ change sign. Clearly, a favorable ΔS almost entirely accounts for the strength of association of Fd with FNR in the physiological temperature range.

ΔC_p and the Area of Buried Surface in the Fd:FNR Complex. From $\Delta C_p = -680 \pm 44$ J mol^{-1} K^{-1}, which is smaller than most values reported in the literature (Sturtevant, 1977; Spolar & Record, 1994; Hibbits et al., 1994), one may estimate the nonpolar surface area, ΔA_{np}, buried in the complex (Spolar & Record, 1994; Livingstone et al., 1991; Murphy et al., 1990; Gill & Wadsö, 1976). The proportionality is

$$\Delta C_p = (0.25 \pm 0.02)\Delta A_{np} \quad (6)$$

where ΔA_{np} has units of Å^2 and ΔC_p has units of cal mol^{-1} K^{-1}. The estimate yields 650 ± 100 Å^2 of nonpolar protein surface. Furthermore, statistical analysis of the distribution of amino acid residues in globular proteins of known 3D structure revealed that, on average, 59% of the solvent accessible surface is nonpolar (Miller et al., 1987). If the above 650 ± 100 Å^2 accounts for 59% of the surface buried in the complex, the total buried area is 1100 ± 300 Å^2. This value is on the same order of magnitude as the buried surface areas in the complex of cytochrome *c* with cytochrome *c* peroxidase (Pelletier & Kraut, 1992)[4] and in the ternary complex composed of methylamine dehydrogenase, amicyanin, and cytochrome c_{551} (Chen et al., 1994). These are the only known crystal structures of electron transfer complexes.

Nature of the Favorable Entropy of Binding. The positive entropy change can be explained by a strong hydrophobic effect. ΔS (Table 1) may be described as (Spolar & Record, 1994)

$$\Delta S = \Delta S_{hydr} + \Delta S_{trans} + \Delta S_{specific}$$

ΔS_{hydr} is the contribution by the hydrophobic effect. ΔS_{trans} accounts for the reduction in the overall rotational and translational degrees of freedom, as well as the immobilization of amino acid side chains at the complex interface. $\Delta S_{specific}$ describes system-specific contributions such as reduction of main chain mobility and entropic contributions from polar interactions. ΔS_{hydr} may be estimated from

$$\Delta S_{hydr} = 1.35\Delta C_p \ln(T/386)$$

(Spolar & Record, 1994) where ΔC_p (in J mol^{-1} K^{-1}) is the measured heat capacity change, T is the absolute temperature, and 386 is the reference temperature at which the entropy of transfer of nonpolar liquids to water vanishes [Spolar and Record (1994) and references therein]. For the Fd:FNR complex at 300 K we obtain $\Delta S_{hydr} = 232$ J mol^{-1} K^{-1} (55 eu). Because $\Delta S = 125$ J mol^{-1} K^{-1}, $\Delta S_{trans} + \Delta S_{specific}$ accounts for about -107 J mol^{-1} K^{-1} (-26 eu). Because of a lack of structural information on the Fd:FNR complex, we cannot provide information about the nature of ΔS_{trans} and $\Delta S_{specific}$, which together oppose binding. However, we may try to assess the nature of the hydrophobic effect.

The Role of Water in the Fd:FNR Complex. It has become a guideline that water is excluded from tightly fitting protein−protein interfaces. Chothia and Janin (1975) proposed that the entropy gain from the dehydration of interacting surfaces is mainly responsible for the thermodynamic stability of protein complexes. To probe for the importance of water in stabilizing the Fd:FNR complex, we measured the binding affinity under conditions of lowered water activity in the presence of glycerol. High osmotic pressure promotes dehydration of the molecular surface and should promote binding if water is released from the complex interface (Rand & Parsegian, 1989; Colombo et al., 1992). If, however, water stabilizes binding, for example by improving the complementarity at the complex interface, a decrease in water activity will decrease the binding affinity. The latter was observed when lysozyme bound to an antibody Fv fragment (Bhat et al., 1994).

We compared K_A at 0%, 26%, and 52% (w/w) glycerol in Tris buffer (pH 7.5). Glycerol was chosen because it does not noticeably change pH, ionic strength, and the macro-

[4] Calculation with coordinates of the complex (Pelletier & Kraut, 1992) using the CFF91 set of atomic radii in the program Delphi, version 2.5 (Biosym Technologies, San Diego, CA). The value of -1150 Å^2 is the sum of the water accessible surfaces of the individual proteins minus the water accessible surface calculated for the complex.

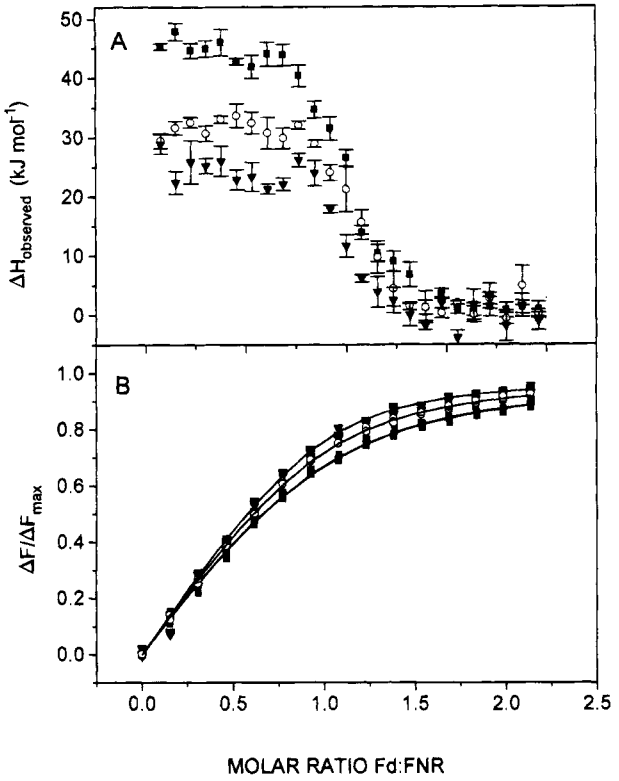

FIGURE 3: Experimental data used to characterize the binding of Fd to FNR under conditions of osmotic stress. Panel A: Reaction isotherms obtained by ITC in 50 mM Tris-HCl (pH 7.5) at 27 °C. Mean ± sem for ≥3 experiments performed in the presence of 0% (■), 26% (○), and 52% (▼) glycerol. Panel B: Quenching of tryptophan fluorescence emission at 347 nm as a function of the molar ratio of Fd to FNR; same conditions and symbols as in panel A. Two experiments were performed at each glycerol concentration. The solid line is the best fit according to eq 1 for the two experiments.

scopic dielectric constant of buffer solutions (Kornblatt et al., 1993). Furthermore, the structure of the proteins was not grossly altered by high concentrations of glycerol. Absorbance spectra remained unchanged for 6 h at the experimental pH, ionic strength, and temperatures from 10 to 40 °C in stirred solutions containing up to 60% glycerol (not shown). Only the intensity of the tryptophan emission spectrum of FNR measured in 52% (w/w) glycerol was lowered by about 5% (not shown).

K_A was measured by ITC and fluorescence quenching (Table 2). Figure 3A depicts the averaged reaction isotherms from ITC experiments at 0%, 26%, and 52% glycerol. The experimental error increased with increasing concentrations of glycerol. Nonetheless, a statistically significant set of parameters could be obtained at each concentration of glycerol. The addition of glycerol clearly favored the association of Fd and FNR. In the ITC experiment, K_A increased from $(6.5 \pm 1.9) \times 10^6$ M^{-1} in the absence of glycerol to $(2.59 \pm 1.58) \times 10^7$ M^{-1} in 52% glycerol. The increase in the fluorescence quenching experiment was somewhat smaller: from $(4.2 \pm 0.2) \times 10^6$ to $(8.2 \pm 0.9) \times 10^6$ M^{-1}. Figure 3B shows the fluorescence quenching curves at different glycerol concentrations.

As mentioned earlier, association constants determined by ITC and fluorescence quenching titration systematically deviated from each other. This observation is intriguing, particularly since the difference became larger with increasing glycerol concentrations (Table 2). We have no satisfying

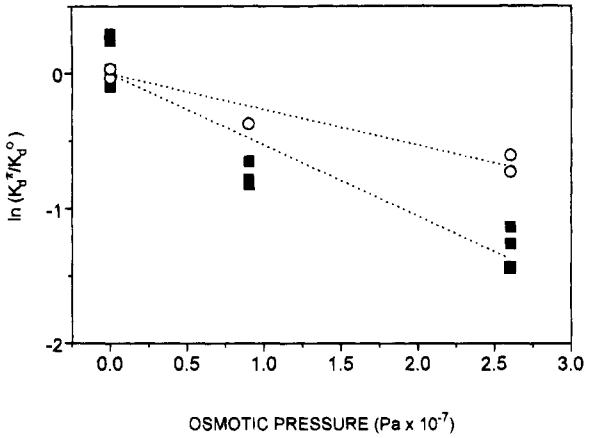

FIGURE 4: Change of stability of the Fd:FNR complex under osmotic stress. Normalized dissociation constants are plotted according to eq 2. Lines are best fits to eq 2 for the data from ITC (■) and fluorescence quenching titration (○).

explanation for this observation except to note that ITC and fluorescence quenching probe for inherently different physicochemical aspects of the binding process.

A striking result of the ITC experiment under osmotic stress conditions was the large apparent negative enthalpy change observed at higher glycerol concentrations (Table 2). A decrease in the apparent enthalpy was unexpected since removal of water from the buried complex interface is thought to be primarily entropic. However, it must be remembered that the apparent net enthalpy change registered in the ITC experiment is a global property of the system and may not be attributed to a particular event alone. Considering the large enthalpy of mixing of glycerol with water, one expects an exothermic reaction if water is squeezed out from the interface into the glycerol-containing bulk solvent. We speculated that the apparent net enthalpies in glycerol are composed of a weakly positive or weakly negative contribution by the binding reaction proper (as in the absence of glycerol; see Table 1) and a larger negative contribution due to the release of bound water from the complex interface into the water/glycerol mixture. To test this hypothesis, we measured K_A at four different temperatures in the presence of 52% glycerol in Tris buffer by the fluorescence quenching method (Table 2). In this way, we could obtain ΔG via direct measurement of K_A, avoiding the problem of water release into the glycerol-containing bulk solvent. With the values for ΔG from fluorescence quenching, and assuming that ΔC_p of association is the same in glycerol and water, we estimated ΔH by van't Hoff analysis. To this end, ΔG was fit to the equation $\Delta G = \Delta H(T^*) + \Delta C_p(T - T^*) - T\{[\Delta H(T^*) - \Delta G(T^*)]/T^* + \Delta C_p \ln(T/T^*)\}$ (Varadarajan et al., 1992). Choosing $T^* = 300$ K and with $\Delta C_p = -680$ cal mol^{-1} K^{-1}, ΔH (300 K) was approximately 2.5 kJ mol^{-1}. This demonstrated that the enthalpy of complex formation in 52% glycerol was also close to zero and that our explanation of an exothermic heat of mixing accompanying the release of water into the glycerol-containing bulk solution is probably correct.

Estimation of the Number of Water Molecules Released from the Complex. According to eq 2, a plot of $\ln(K_d^{\pi}/K_d^{\circ})$ against the change in osmotic pressure yields the volume of the osmotically labile water, ΔV_w. The plot is shown in Figure 4. From the slope one calculates $\Delta V_w = 126 \pm 26$ mL mol^{-1} (ITC data) or 70 ± 36 mL mol^{-1} (fluorescence

data). Taking 18 mL mol^{-1} for the partial molal volume of water, the number of released water molecules is 7 ± 1 (ITC data) or 4 ± 2 (fluorescence data). How do these numbers compare to the estimated ΔS_{hydr}, which is thought to account primarily for the release of water upon formation of a protein−protein complex (Spolar & Record, 1994)? Dunitz (1994) has argued that the entropy of water tightly bound to the surface of a protein may resemble the entropy of ice or a crystalline hydrate. This sets an upper limit to the gain of entropy of about 28 J mol^{-1} K^{-1} when water is released from the protein surface (Dunitz, 1994). With this estimate, and assuming that the entire ΔS_{hydr} of 232 J mol^{-1} K^{-1} (see above) accounts for the release of water from the interface, we obtain a minimum of eight water molecules per Fd:FNR complex, which is in very good agreement with the measured ΔV_w. This calculation is, however, based on several unproven assumptions. For example, one assumes that bound and free water are of the same density and that the hydration shell around the molecule is inaccessible to the cosolvent glycerol. This is not likely as some of the bound water may already be released when the free proteins are transferred from buffer into glycerol/buffer mixtures, i.e., before the association process. That glycerol changes the solvation of the free proteins in a nonlinear way could explain why in Figure 4 the relative dissociation constant of the complex, K_d^{π}/K_d°, decreases more from 0% to 26% glycerol than from 26% to 52% glycerol. From all of this it follows that exclusion of 4−7 water molecules is only a lower estimate accounting for the most tightly bound water molecules. Indeed, by assuming that $\Delta C_p = -680 \pm 44$ kJ mol^{-1} K^{-1} accounts for most of the hydrophobic effect (Sturtevant, 1977) and that the decrease in heat capacity per mole of water lost is, on average, 24 J mol^{-1} K^{-1} (Suurkuusk, 1974), one calculates the release of 25−28 water molecules.

There is one other estimate of water release in a protein−protein complex. Kornblatt et al. (1993), also using the osmotic stress method, found that binding of cytochrome b_5 to porphyrin−cytochrome c is accompanied by a volume change of −47 mL mol^{-1}, which corresponds to the exclusion of about three water molecules. A similar or somewhat larger volume change for the same complex was deduced by the hydrostatic pressure method (Kornblatt et al., 1988; Rodgers et al., 1988). Release and rebinding of water accompany some enzymatic reactions (Kornblatt & Hoa, 1990; Rand et al., 1993).

The complex composed of cytochrome c_3 and ferredoxin I from *Desulfovibrio desulfuricans* is the only other electron transfer complex studied by calorimetry (Guerlesquin et al., 1987). This complex is also stabilized by a large positive entropy term, the enthalpy term being unfavorable. Also, for the cytochrome b_5−cytochrome c complex there is indirect evidence for entropic stabilization, although the complex was not analyzed by calorimetry (Mauk et al., 1982). As mentioned in the introduction, these and other electron transfer complexes are characterized by electrostatic bonds. One is tempted to speculate that a favorable entropy of binding is typical for electron transfer complexes. The release of water densely clustered around charged groups in the free proteins may be a source of entropy gain. Decreasing the water activity will not only promote desolvation of the protein surfaces but also facilitate the formation of charge bridges.

CONCLUSIONS

1. The thermodynamic parameters of binding of Fd to FNR were determined by isothermal titration calorimetry. In the physiological temperature range, the binding is entropically driven, indicating a strong contribution from hydrophobic effects. The observed heat capacity change is negative but relatively small. Using published correlations between the heat capacity change and the burial of nonpolar surface area, we could estimate the surface area that is buried in the Fd:FNR complex. Our estimate is in rough agreement with values published for two other electron transfer complexes for which X-ray data are available.

2. Uptake of a proton makes an important enthalpic contribution to the stability of the Fd:FNR complex. Although the nature of the protonated group remains ambiguous, the present results and those of our previous studies suggest that His-19 or His-90 of FNR may become protonated in the complex.

3. High osmotic pressure promotes the binding of Fd to FNR. This strongly indicates that the hydrophobic effect is due to the release of water molecules when Fd and FNR associate. Exclusion of 4−7 water molecules, probably a lower estimate, was calculated from the volume change accompanying association at high osmotic pressure.

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