An Aromatic Amino Acid Is Required at Position 65 in *Anabaena* Ferredoxin for Rapid Electron Transfer to Ferredoxin:NADP$^+$ Reductase

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Abstract: Vegetative cell ferredoxin (Fd) from the cyanobacterium *Anabaena* 7120 functions in photosynthesis to transfer electrons from photosystem I to ferredoxin:NADP$^+$ reductase (FNR). Previous work using site-directed mutagenesis and laser flash photolysis (Hurley et al., *Biochemistry* **1993**, *32*, 9346–9354) has shown that replacing Phe-65 with aliphatic amino acids decreased the rate constant for the Fd to FNR electron-transfer (ET) reaction by more than 3 orders of magnitude, while leaving other properties of the protein (UV–vis and CD spectra and reduction potential, etc.) intact. The present study demonstrates that replacing Phe-65 with either of the aromatic residues Trp or Tyr restores wild type (wt) activity with regard to ET to FNR. These mutants also restored wt ionic strength dependencies of the Fd to FNR ET rate constant, which, for the aliphatic Phe-65 mutants, were vastly different from those of the wt. In addition, a double mutant which places an aliphatic residue at position 65 and an aromatic residue at an adjacent site also reacted more than 4 orders of magnitude slower in ET to FNR. The observed rate constant for ET to FNR of the Phe-65 to Ala-65 mutant was found to be independent of FNR concentration, indicating that this rate constant reflects processes occurring during intracomplex ET between the two proteins. These results clearly demonstrate that Fd from *Anabaena* 7120 requires an aromatic amino acid at position 65 for efficient ET to FNR.

Introduction

Considerable research has been done in an effort to understand protein–protein electron-transfer (ET) reactions, especially concerning the effects of thermodynamic driving force, the distance and relative orientation of redox centers, and the role of the intervening protein matrix.[1] In a previous series of experiments, we have identified two amino acid residues E94 and F65 in *Anabaena* ferredoxin (Fd) that are essential for ET to *Anabaena* ferredoxin:NADP$^+$ reductase (FNR).[2] The possibility that aromatic residues can facilitate protein ET reactions has been both supported[3] and rejected[4] by various studies. Thus, an aromatic residue at the C-terminal position in putidaredoxin is required for optimal ET to cytochrome $P450_{cam}$.[3a,b] Replacing this Trp with aliphatic amino acids decreased the first-order ET rate constant by factors of 4–8; effects on protein binding were suggested as being the cause of this effect. Farver and Pecht[3c] have implicated Trp-48 in ET reactions involving azurin, although a theoretical analysis[4a] has concluded that it is the peptide backbone rather than the aromatic π system that provides the necessary orbital overlap and coupling between the redox centers. In yeast cytochrome *c* peroxidase (CCP), the mutant W191F showed an approximately (5×10^3)-fold decrease in the rate constant for the H_2O_2-dependent oxidation of ferrocytochrome *c* compared to that for the wild type (wt) CCP.[4b] Similarly, with yeast flavocytochrome b_2, the mutant Y143F showed a 20-fold decrease in intramolecular ET during enzyme turnover.[4c,5] In these latter two cases, however, replacing one aromatic residue with another did not restore wt behavior, indicating that aromaticity *per se* was not responsible for the ET efficiency of the wt protein.

Fds are a class of small, acidic, low-potential iron–sulfur redox proteins that function in photosynthetic ET, N_2 fixation, and carbon and sulfur metabolism.[6] In photosynthesis, a [2Fe–2S] Fd is the terminal electron acceptor from photosystem I and the electron donor to FNR, which catalyzes the reduction of NADP$^+$ to NADPH. The structure of the Fd from the cyanobacterium *Anabaena* 7120 has recently been solved to 2.5-Å resolution[7] and refined to 1.9 Å, thus making this protein a good candidate for studies of protein structure–function relationships. The experiments reported herein, utilizing site-directed mutagenesis in

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(1) (a) Marcus, R. A.; Sutin, N. *Biochim. Biophys. Acta* **1985**, *811*, 265–322. (b) Winkler, J. R.; Gray, H. B. *Chem. Rev.* **1992**, *92*, 369–379. (c) Beratan, D. N.; Onuchic, J. N.; Winkler, J. R.; Gray, H. B. *Science* **1992**, 1740–1741. (d) Wuttke, D. S.; Bjerrum, M. J.; Winkler, J. R.; Gray, H. B. *Science* **1992**, 1007–1009. (e) Moser, C. C.; Keske, J. M.; Warncke, K.; Farid, R. S.; Dutton, P. L. *Nature* **1992**, *355*, 796–802. (f) McLendon, G. *Acc. Chem. Res.* **1988**, *21*, 160–167.

(2) Hurley, J. K.; Salamon, Z.; Meyer, T. E.; Fitch, J. C.; Cusanovich, M. A.; Markley, J. L.; Cheng, H.; Xia, B.; Chae, Y. K.; Medina, M.; Gomez-Moreno, C.; Tollin, G. *Biochemistry* **1993**, *32*, 9346–9354.

(3) (a) Davies, M. D.; Qin, L.; Beck, J. L.; Suslick, K. S.; Koga, H.; Horiuchi, T.; Sligar, S. G. *J. Am. Chem. Soc.* **1990**, *112*, 7396–7398. (b) Davies, M. D.; Sligar, S. G. *Biochemistry* **1992**, *31*, 11383–11389. (c) Farver, O.; Pecht, I. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 6968–6972. (d) Axup, A. W.; Albin, M.; Mayo, S. L.; Crutchley, R. J.; Gray, H. B. *J. Am. Chem. Soc.* **1988**, *110*, 435–439. (e) Poulos, T. L.; Kraut, J. *J. Biol. Chem.* **1980**, 10322–10330. (f) Pelletier, H.; Kraut, J. *Science 258*, 1748–1755.

(4) (a) Broo, A.; Larsson, S. *J. Phys. Chem.* **1991**, *95*, 4925–4928. (b) Mauro, J. M.; Fishel, L. A.; Hazzard, J. T; Meyer, T. E.; Tollin, G.; Cusanovich, M. A.; Kraut, J. *Biochemistry* **1988**, 6243–6256. (c) Miles, C. S.; Rouviere-Fourmy, N.; Lederer, F.; Mathews, F. S.; Reid, G. A.; Black, M. T.; Chapman, S. K. *Biochem. J.* **1992**, *285*, 187–192. (d) Concar, D. W.; Whitford, D.; Pielak, G. J.; Williams, R. J. P. *J. Am. Chem. Soc.* **1991**, *113*, 2401–2406. (e) Everest, A. M.; Wallin, S. A.; Stemp, E. D. A.; Nocek, J. M.; Mauk, A. G.; Hoffman, B. M. *J. Am. Chem. Soc.* **1991**, *113*, 4337–4338. (f) Pielak, G. J.; Mauk, A. G.; Smith, M. *Nature* **1985**, 152–154.

(5) Tollin, G.; Hurley, J. K.; Hazzard, J. T.; Meyer, T. E. *Biophys. Chem.* **1993**, in press.

(6) (a) Knaff, D. B.; Hirasawa, M. *Biochim. Biophys. Acta* **1991**, *1056*, 93–125. (b) Lovenberg, W., Ed. *Iron-Sulfur Proteins*; Academic Press: New York, Vol. I, 1973; Vol. II, 1974; Vol. III, 1977.

(7) Rypniewski, W. R.; Breiter, D. R.; Benning, M. M.; Wesenberg, G.; Oh, B.-H.; Markley, J. L.; Rayment, I.; Holden, H. M. *Biochemistry* **1991**, *30*, 4126–4131.

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conjunction with laser flash photolysis/time-resolved absorption spectroscopy, demonstrate that the amino acid residue at position 65 in *Anabaena* Fd must be aromatic in order to allow rapid electron transfer to *Anabaena* FNR, its physiological redox partner. Thus, Trp and Tyr are as effective as Phe, but the aliphatic residues Ala and Ile are essentially inactive. Trp and Tyr also restore wt ionic strength dependence, which for the aliphatic mutants at position 65 is dramatically different than the dependence shown by the wt protein. We also demonstrate in this study that the inactivity shown by the aliphatically substituted mutants is a consequence of an impairment of the intracomplex ET process.

Experimental Section

A full description of the subcloning, mutagenesis, protein overexpression, cluster reconstitution, and protein purification methods employed in making *Anabaena* 7120 mutants at the University of Wisconsin will be presented elsewhere (H. Cheng, B. Xia, J. L. Markley, manuscript in preparation). Briefly, the petF gene[8] from plasmid pAn662 was cloned into plasmid pET9a (Novogen, Madison, WI) containing the T7 promoter by using the *Nde* I and *Bam*H I restriction sites. The resulting construct pET9a/F was placed in *Escherichia coli* host BL21(DE3)/pLysS. Protein expression was induced by adding isopropylthiogalactoside (IPTG) into the growth culture when $OD_{600} = 1.2$ was reached. After harvesting, the cells were lysed using a freeze–thaw cycle and the protein was reconstituted by a modification of the method of Coghlan and Vickery.[9]

Mutants were prepared by oligonucleotide-directed mutagenesis of recombinant M13mp 18 DNA with the pET9a/F plasmid fragment inserted between the *Eco*R I and *Sal* I restriction sites. The Kunkel method of single-strand DNA site-directed mutagenesis was used as described.[10,11] The mutated DNAs were subcloned back into the pET9a vector. The resulting mutated recombinant plasmids were sequenced by a double-stranded dideoxy method[12] to confirm that mutagenesis was limited to the predicted sites.

FNR from *Anabaena* strain 7119 was isolated and purified as previously described.[13]

Protein extinction coefficients and methods used to obtain absorption and circular dichroism spectra are as previously reported.[2] The laser flash photolysis system utilized a nitrogen laser-pumped dye laser,[2] and the optical system used to monitor the reaction kinetics has been described,[14,15] as has the photochemical reaction by which the 5-deazariboflavin (dRf) triplet state initiates protein–protein ET.[16] Briefly, the laser-generated dRf triplet abstracts a hydrogen atom from EDTA which is present in large excess. The resulting dRf semiquinone (dRfH) then reduces oxidized protein in competition with its own disproportionation. All kinetic experiments were performed under pseudo-first-order conditions, in which protein is present in large excess over the dRfH generated by the laser flash (<1 μM). Under these conditions, dRfH reacts almost exclusively with oxidized Fd when both Fd and FNR are present simultaneously.[17] This allows ET from reduced Fd to oxidized FNR to be monitored (see below). Solutions of dRf (95–100 μM in 4 mM phosphate buffer, pH 7.0, containing 1 mM EDTA) were made anaerobic by bubbling for 1 h with H_2O-saturated argon. Microliter volumes of concentrated protein solutions were introduced into this solution in a 1-cm cuvette through a rubber septum, and Ar gas was blown over the sample surface to remove any added oxygen. Generally, data from four to eight laser flashes were averaged. Ionic strength was adjusted by adding aliquots of 5 M NaCl. Kinetic traces were analyzed using a computer fitting routine (Kinfit, OLIS Co., Bogart, GA).

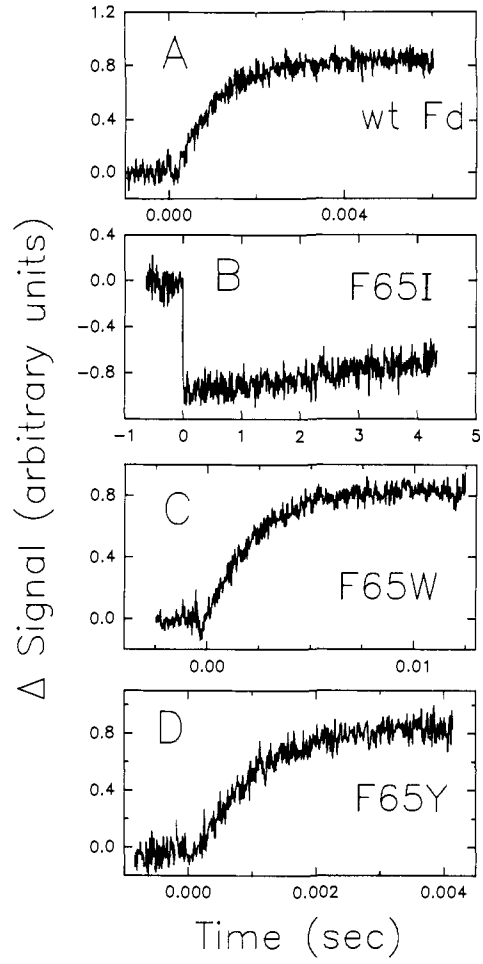

Figure 1. Transient decays of dRf(100 μM)/EDTA(1 mM)/Fd/FNR solutions in 4 mM phosphate buffer, pH 7.0: (A) 40 μM wt Fd + 5 μM FNR, 600 nm; (B) 40 μM F65I mutant Fd + 10 μM FNR, 507 nm; (C) 40 μM F65W mutant Fd + 5 μM FNR, 600 nm; and (D) 40 μM F65Y mutant Fd + 5 μM FNR, 600 nm. Data are shown at 507 nm for F65I because the absorbance change at 600 nm is quite small on this time scale.

Results and Discussion

In previous work,[2] we have used laser flash photolysis to investigate the kinetics of reduction of FNR by wt and several site-directed mutants of Fd. This reaction was shown to proceed via a two-step mechanism[2,17] as depicted in eq 1.

$$Fd_{red} + FNR_{ox} \overset{K_d}{\rightleftarrows} (Fd_{red}{:}FNR_{ox}) \overset{k_{et}}{\rightleftarrows} Fd_{ox} + FNR_{red} \quad (1)$$

Fd_{red} is produced *in situ* by photochemical generation of 5-deazariboflavin semiquinone (dRfH).[16] All mutant Fds investigated to date (including the new mutants reported in the present study; cf. Table I) are reduced by dRfH with rate constants differing by no more than a factor of 2 from those of wt Fd,[2] indicating that the intrinsic ET properties of the [2Fe–2S] cluster are not appreciably perturbed (see below). Single mutations at seven different residue positions also resulted in wt behavior with respect to ET to FNR, whereas mutations at two other sites (E94K and F65A or F65I) each caused a decrease of approximately 4 orders of magnitude in this ET rate constant.[2]

Figure 1A shows a transient decay corresponding to reduction of FNR by wt Fd_{red}, measured at 600 nm, a wavelength which monitors the reduction of the FAD cofactor of FNR. The second-order rate constants (k_2, which reflects both K_d and k_{et}[2]) for wt

(8) Alam, J.; Whitaker, R. A.; Krogman, D. W.; Curtis, S. E. *J. Bacteriol.* **1986**, *168*, 1265–1271.
(9) Coghlan, V. M.; Vickery, L. E. *J. Biol. Chem.* **1991**, *266*, 18606–18612.
(10) Kunkel, T. A.; Roberts, J. D.; Zakour, R. A. *Methods Enzymol.* **1987**, *154*, 367–372.
(11) Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., Struhl, K., Eds. *Current Protocols in Molecular Biology*; John Wiley & Sons: New York, 1989.
(12) Lim, H. M.; Pene, J. J. *Gene Anal. Tech.* **1988**, *5*, 32–39.
(13) Pueyo, J. J.; Gomez-Moreno, C. *Prep. Biochem.* **1991**, *21*, 191–204.
(14) Przysiecki, C. T.; Bhattacharyya, A. K.; Tollin, G.; Cusanovich, M. A. *J. Biol. Chem.* **1985**, *260*, 1452–1458.
(15) Bhattacharyya, A. K.; Tollin, G.; Davis, M.; Edmondson, D. E. *Biochemistry* **1983**, *22*, 5270–5279.
(16) Tollin, G.; Hazzard, J. T. *Arch. Biochem. Biophys.* **1991**, *287*, 1–7.
(17) Walker, M. C.; Pueyo, J. J.; Navarro, J. A.; Gomez-Moreno, C.; Tollin, G. *Arch. Biochem. Biophys.* **1991**, *287*, 351–358.

Table I. Rate Constants for Reduction of Anabaena Fd by dRfH and for Oxidation of Anabaena Fd by Anabaena FNR[a]

	$k \times 10^{-8}$, M^{-1} s^{-1}	
	dRfH	FNR
wt[b]	2.2 ± 0.2	1.2 ± 0.1
F65I[b]	1.1 ± 0.1	~0.00002
F65A[b]	1.2 ± 0.2	~0.00007[c]
F65W	1.2 ± 0.2	0.9 ± 0.1
F65Y	1.5 ± 0.2	1.3 ± 0.2
S64Y/F65A	1.6 ± 0.1	~0.00005

[a] Rate constants were measured at an ionic strength of 12 mM. [b] Taken from ref 2. [c] This rate constant has been shown to be first order with a value of 0.6 s^{-1} (see text).

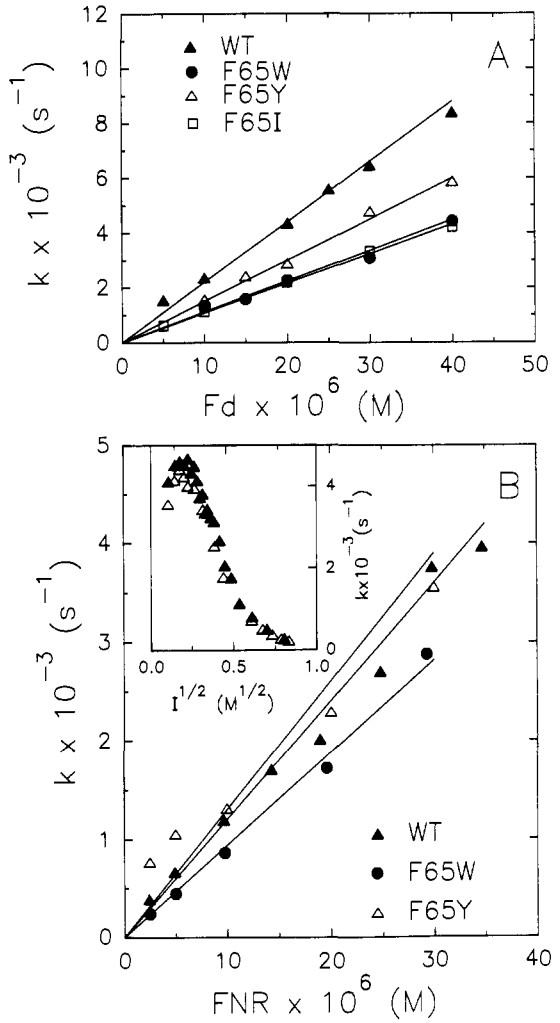

Figure 2. (A) Second-order plots for the reduction of wt, F65I, F65W, and F65Y Fds by dRfH. Kinetics were monitored at 465 nm. Conditions were as described in Figure 1. (B) Pseudo-first-order rate constants for reduction of FNR by wt and mutant Fds. Varying concentrations of FNR were titrated into solutions containing 30 μM wt Fd, 40 μM F65W, or 40 μM F65Y. Kinetics were monitored at 600 nm. Conditions were as described in Figure 1. Inset: ionic strength dependence of reduction of FNR (30 μM) by wt (30 μM) and F65Y (40 μM) Fds. Ionic strength was adjusted with NaCl.

and a series of F65 mutant Fds are given in Table I. Figure 1B shows a kinetic transient decay for the mutant F65I obtained at 507 nm, which monitors reoxidation of Fd_{red} by FNR.[2] Rate constants obtained at these two wavelengths are identical within experimental error, as would be expected for ET from Fd_{red} to FNR_{ox}. The k_2 for this reaction with the F65I mutant is approximately 60 000 times smaller than that for wt Fd. A similar result is obtained for F65A, as shown in Table I. Note that these effects are considerably larger than those reported for putidaredoxin.[3a,b] These mutations did not affect the gross protein structure or the environment of the Fe–S cluster, as indicated by UV–vis and CD spectroscopy.[2] Furthermore, the reduction potential of these mutants, their reaction with dRfH, and their ability to form complexes with FNR were closely comparable to those of wt Fd.[2] We therefore conclude that these mutations directly influence the ET process (see below for further discussion).

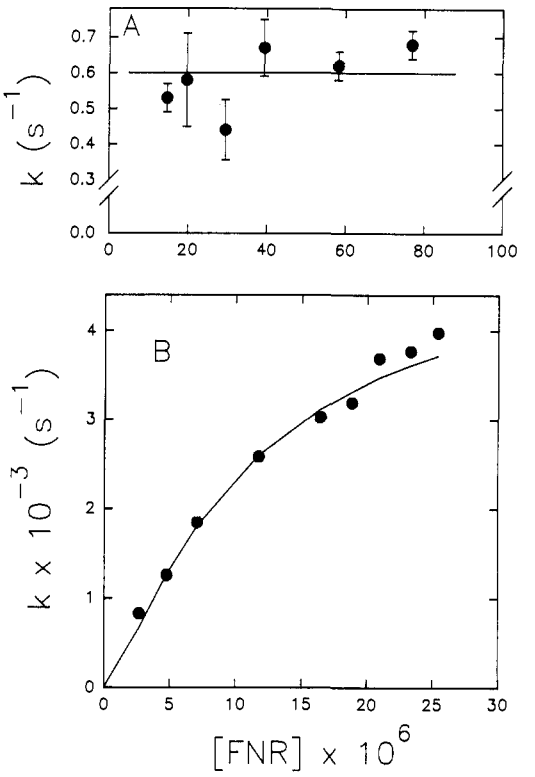

Figure 3. (A) FNR concentration dependence of the rate constant for ET from Fd mutant F65A to FNR. Ionic strength was 100 mM. Data were collected at 507 nm. Other conditions were as described in the Experimental Section. (B) FNR concentration dependence of the rate constant for ET from recombinant wt Fd to FNR. Conditions were as described in (A) except that decays were monitored at 600 nm. Data in (B) are from ref 2.

In order to determine whether or not the aromatic character of the residue at position 65 was the critical parameter in controlling reactivity, the mutants F65W and F65Y were prepared. Like all of the other Fds studied thus far, both mutant proteins reacted with dRfH with rate constants within a factor of 2 of that for wt Fd (Table I; second-order plots shown in Figure 2A). Figures 1C and 1D show kinetic traces for reduction of FNR by reduced F65W and F65Y, respectively. Second-order plots for the latter reactions are shown in Figure 2B. These plots are reproducibly linear at low ionic strengths within our experimental limits (see below). It is clear from these results that both of these mutants show essentially wt ET behavior toward dRfH and FNR (Table I). The inset to Figure 2B further shows that replacement of F65 by another aromatic residue also yields wt behavior with respect to the effect of ionic strength on the kinetics of ET to FNR, a property which differs greatly for F65 mutants containing aliphatic substitutions.[2] For the ET-impaired mutants F65A, F65I, and E94K, increasing the ionic strength (up to 1 M) resulted in a monotonic increase in k_{obs}. The explanation for this was that interprotein orientations in the complex were so far from optimal for efficient ET that any "loosening" of the complex by increasing the ionic strength allowed the proteins to assume orientations which were more favorable for ET. In contrast, wt behavior, also shown by mutants F65W and F65Y (cf. inset to Figure 2B), is characterized by small increases in the rate constant at low ionic strengths followed by decreases in this parameter as the ionic strength is increased

further. The initial increase in k_{obs} with ionic strength in the low-ionic-strength region has also been observed previously for the *Anabaena* 7119 proteins[17] and has been seen in other protein–protein electron-transfer systems including cytochrome *c*/cytochrome b_5,[18] cytochrome *c*/cytochrome *c* oxidase, and cytochrome *c*/cytochrome *c* peroxidase[19,20] (Hazzard et al., 1988, 1991). In these previous studies, this effect was attributed to a situation in which the electrostatically most stable complex at low ionic strength is not optimal for electron transfer and that some "loosening" of the complex is necessary to allow the two proteins to assume a mutual orientation that is more favorable to electron transfer. At higher ionic strengths, the normal behavior for an interaction between proteins of opposite charge is observed. In the earlier study with nonrecombinant *Anabaena* 7119 Fd and FNR,[17] the results clearly demonstrated that the value of k_{ET} for the intracomplex reaction had an optimal value at intermediate ionic strengths.

It has been noted[2] that recombinant wt *Anabaena* Fd does not show saturation kinetics in an accessible concentration range at low ionic strengths, whereas native wt Fd does.[17] Saturation kinetics are observed, however, at an ionic strength of 100 mM, as is shown in Figure 3B[2] for wt Fd. The reason for this difference is unclear at present. Whatever the cause, in order to obtain saturation and thereby sort out possible effects due to K_d and k_{ET}, we performed an experiment with the ET-impaired mutant F65A and FNR at I = 100 mM. As shown in Figure 3A, we find that the observed rate constant is independent of FNR concentration. This provides strong evidence that intracomplex ET is the rate-limiting step in the F65A/FNR system. The value for k_{ET} obtained from these data is 0.6 ± 0.1 s^{-1}, which is 4 orders of magnitude smaller than that obtained for wt Fd from analysis of the data in Figure 3B (5100 s^{-1}, cf. ref 2). We presume, on the basis of their analogous properties, a similarly small, rate-determining k_{ET} for F65I, although this has not been determined directly. Thus, removal of the aromatic character of the side chain at position 65 clearly impairs the ability of Fd to transfer an electron to FNR.

In addition, we have also made the double mutant S64Y/F65A to test the possibility that an aromatic residue adjacent to position 65 would restore wt reactivity. However, like the ET-impaired F65 mutants, S64Y/F65A reacted comparably to wt Fd toward dRfH and reacted more than 4 orders of magnitude more slowly in ET to FNR (Table I; data not shown). The ionic strength dependence of the reaction rate constant with this double mutant was also similar to the F65A and F65I mutants (not shown).

There are two possible explanations for the above results: (i) the steric properties of an aromatic ring are required to achieve an appropriate orientation of the two proteins during intracomplex ET and (ii) the π electron system of the aromatic ring is involved in electronically coupling the two protein redox cofactors. Although the present results cannot distinguish between these possibilities, it is noteworthy that the surface of FNR which contains the FAD cofactor does not have any exposed aromatic residues which might participate in a stacking interaction with the aromatic ring at position 65, although there is a group of hydrophobic residues located near the edge of the FAD isoalloxazine ring (J. Fontecilla, M. Frey, L. Serre, personal communication). Further work will be required to clarify this issue.

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(18) Hazzard, J. T.; McLendon, G.; Cusanovich, M. A.; Das, G.; Sherman, F.; Tollin, G. *Biochemistry* **1988**, *27*, 4445–4451.

(19) Hazzard, J. T.; Rong, S. Y.; Tollin, G. *Biochemistry* **1991**, *30*, 213–222.

(20) Meyer, T. E.; Rivera, M.; Walker, F. A.; Mauk, M. R.; Mauk, A. G.; Cusanovich, M. A.; Tollin, G. *Biochemistry* **1993**, *32*, 622–627.