Differential Contributions of NADPH-Cytochrome P450 Oxidoreductase FAD Binding Site Residues to Flavin Binding and Catalysis

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Transfer of reducing equivalents from NADPH to the cytochromes P450 is mediated by NADPH-cytochrome P450 oxidoreductase, which contains stoichiometric amounts of tightly bound FMN and FAD. Hydrogen bonding and van der Waals interactions between FAD and amino acid residues in the FAD binding site of the reductase serve to regulate both flavin binding and reactivity. The precise orientation of key residues (Arg454, Tyr455, Cys472, Gly488, Thr491, and Trp677) has been defined by x-ray crystallography (Wang, M., Roberts, D. L., Paschke, R., Shea, T. M., Masters, B. S., Kim, J.-J. P. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 8411–8416). The current study examines the relative contributions of these residues to FAD binding and catalysis by site-directed mutagenesis and kinetic analysis. Mutation of either Tyr456, which makes van der Waals contact with the FAD isoalloxazine ring and also hydrogen-bonds to the ribityl 4'-hydroxyl, or Arg454, which bonds to the FAD pyrophosphate, decreases the affinity for FAD 800- and 25,000-fold, respectively, with corresponding decreases in cytochrome c reductase activity. In contrast, substitution of Thr491, which also interacts with the pyrophosphate group, has a relatively modest effect on both FAD binding (100-fold decrease) and catalytic activity (2-fold decrease), while the G488L mutant exhibited, respectively, 800- and 50-fold decreases in FAD binding and catalytic activity. Enzymic activity of each of these mutants could be restored by addition of FAD. Kinetic properties and the FMN content of these mutants were not affected by these substitutions, with the exception of a 3-fold increase in Y456S Km c and a 70% decrease in R454E FMN content, suggesting that the FMN- and FAD-binding domains are largely, but not completely, independent. Even though Trp677 is stacked against the re-face of FAD, suggesting an important role in FAD binding, deletion of both Trp677 and the carbonyl-terminal Ser678 decreased catalytic activity 50-fold without affecting FAD content.

The flavoprotein NADPH-cytochrome P450 oxidoreductase, which mediates the transfer of electrons from NADPH to the cytochromes P450 and other microsomal electron acceptors, is one of a family of FMN- and FAD-containing enzymes (1–4) that includes the bacterial sulfi te reductase a-subunit and P450-BM3 (5, 6) as well as the mammalian enzymes nitric-oxide synthase and methionine synthase reductase (7, 8). Distinct structural domains involved in substrate, FMN, FAD, and NADPH binding identified by sequence homology (1–4) have been characterized by mutagenesis (9–13) and structural studies (14–16). An interflavin connecting region (amino acids 229–446) with no sequence similarity to any known flavoprotein (1, 2) was postulated to play a role in orienting the two flavin centers for optimal electron transfer. X-ray crystallographic studies confirmed this hypothesis, demonstrating that this interconnecting region is organized into a unique structural domain that interacts with both flavin binding regions and appears to be responsible for aligning the isoaflavoxazine rings end to end with the dimethylbenzene rings facing each other at a spacing of approximately 4 Å (15).

Unlike FMN, FAD is tightly bound to the reductase (17–19), and a number of contacts between the protein and FAD are observed in the crystal structure (15). Residues comprising the FAD binding site include Y455YSIASS461, I471CAVAVEY478, GVAT481, and Trp677 (Fig. 1). The isoaflavoxazine ring of FAD is sandwiched between two aromatic groups, with Trp677 stacked against the re-face and Tyr456 positioned at a 60° angle to the si-face, an arrangement similar to that of the FMN binding site (15). Main chain atoms of Ser457, Tyr455, Cys472, and Val474 are also within hydrogen bonding distance of the isoaflavoxazine ring. The phenolic hydroxyl of Tyr456 forms a hydrogen bond with the ribityl 4'-hydroxyl, with Arg454 and Tyr455 main chain atoms also interacting with the ribityl moiety. Residues Cys472 through Tyr478 lie nearly parallel to the ribityl pyrophosphate, while the negative charge of the pyrophosphate is stabilized by the Arg454 and Thr491 side chains and the polypeptide backbone of residues 489–491 of the N-helix.

The current study utilizes site-directed mutagenesis and kinetic analysis to evaluate the relative contributions of these residues to FAD binding and catalysis. Substitutions within the FAD binding site produced 1.7–300-fold decreases in cytochrome c reductase activity and 100–25,000-fold decreases in FAD binding. Although the FMN- and FAD-binding domains appear to be structurally autonomous, evidence is presented for interactions between the two domains, possibly via the interconnecting domain. Finally, unlike FMN, FAD binding does not require the presence of an aromatic group stacked against the re-face of the isoaflavoxazine ring.

MATERIALS AND METHODS

Expression and purification of recombinant NADPH-cytochrome P450 oxidoreductase were carried out as described previously (9), with the exception that cultures were grown and induced at 28 °C, and the preparation of spheroplasts was omitted. Protein was assayed by the BCA method (20). Reactions for measurement of cytochrome c reductase activity (0.27 μM potassium phosphate, pH 7.7, 50 μM NADPH, and 65 μM cytochrome c) were carried out at 28 °C and were initiated by the addition of protein. The formation of reduced cytochrome c was monitored at 550 nm on a Beckman DU7500 spectrophotometer. For deter-
mination of rates of flavin dissociation, proteins were diluted into either 0.27 or 0.01 mM potassium phosphate, pH 7.7, to a final concentration of 0.2 μg/ml. Incubation at 28 °C for the indicated times, reactions were initiated by the addition of NADPH (50 μM) and cytochrome c (65 μM), and initial rates were determined.

Reconstitution of FAD-deficient proteins with FAD was carried out by incubation with a 2-fold molar excess of FAD for 30 min. Unbound FAD was removed by diluting the samples 10-fold with 50 mM Tris-HCl, pH 7.7, 0.1 mM EDTA, 0.1 mM dithiothreitol, 10% glycerol, followed by centrifugation through a Centricon 50,000 molecular weight cut-off filter.

FMN and FAD content was determined by boiling protein samples for 5 min in the dark, followed by centrifugation at 14,000 × g for 10 min to remove coagulated protein. The absorbance of the released flavins was measured at 450 nm, and the FMN/FAD ratio was determined by measurement of fluorescence at pH 7.7 and 2.6 (excitation at 450 nm, emission at 535 nm) (21).

RESULTS

Mutational analysis of the FAD binding domain provides valuable information on the role of specific amino acid side chains in flavin binding and in maintaining the proper orientation for reduction and electron transfer. Fig. 2 presents the effects of FAD binding site substitutions on flavin content. R454E and Y456S mutants exhibited the greatest changes in FAD binding, with the FAD contents of individual preparations ranging from undetectable to approximately 0.1 mol of FAD/mol of enzyme. Introduction of the bulky leucyl side chain at position 488 also produced a dramatic decrease in FAD content (~80%), while replacement of Thr491 with valine reduced FAD content by approximately 50%. FAD incorporation was unaffected by the C472T, W677Y, S678X (Ser678 deleted), and W677X (Trp677 and Ser678 deleted) mutations.

With the exception of R454E, FAD binding site substitutions did not affect FMN incorporation. Introduction of a negative charge at position 454 had the additional effect of decreasing FMN content by approximately 70%, while replacement of Tyr456 on the si-face of FAD with serine blocked FAD binding but had no effect on FMN content. The marked decrease in R454E FMN content suggests that an interaction between the two flavin domains, responsible for stabilizing FMN binding, may have been disrupted.

Removal of the carboxyl-terminal residue, Ser678, of NADPH-cytochrome P450 oxidoreductase had no effect on the catalytic activity or kinetic properties of the reductase (Table I). Although we were able to express and purify the W677Y and the W677X proteins and evaluate their flavin contents and enzymatic properties, both proteins exhibited a decreased affinity for 2',5'-ADP-Sepharose, suggesting that modifications of the carboxyl-terminal region of the reductase may lead to changes in protein conformation similar to that seen with ferredoxin-NADP⁺ reductase (22).

With the exception of mutations involving Trp677, cytochrome c reductase activities paralleled the observed decreases in flavin content (Table I). Substitutions of Arg454 and Tyr456 produced the largest decreases in enzymic activity (338- and 250-fold, respectively), while introduction of a bulky leucyl side chain at Gly488 had a considerably reduced effect (42-fold). The approximate 50% decrease in FAD content of T491V correlated with a similar decrease in cytochrome c reductase activity, while substitution of Cys472 with threonine or deletion of Ser678 had little or no effect on activity. Although amino acids at the carboxyl terminus of the reductase do not bind FAD directly, the carboxyl-terminal region appears to be important in catalysis and in maintaining an active protein conformation. Thus, the W677Y substitution produced a modest decrease (37%) in specific activity, while deletion of Trp677 and Ser678 decreased activity by 97%, with no change in flavin content.

Additional kinetic properties of the FAD binding site mutants are presented in Table II. The R454E, G488L, and T491V constructs exhibited markedly reduced catalytic activities without affecting either $K_m^{NADPH}$ or $K_m^{cyt}$, consistent with the premise that the primary alteration in these mutants is associated with FAD binding. Conversion of Tyr456, situated at the si-face of the isalloxazine ring, to serine increased $K_m^{cyt}$, while all substitutions involving Trp677, which is stacked against the re-face of the flavin, were associated with decreases in $K_m^{NADPH}$ but did not significantly alter $K_m^{cyt}$.

The addition of FAD to those mutants deficient in this flavin resulted in either the partial or full restoration of cytochrome c reductase activities (Table II). FAD restored full cytochrome c reductase activity to the Y456S and T491V mutants, while activities of the R454E and G488L mutants were restored to approximately 50% of wild type. The dependence of cytochrome c reductase activity on FAD concentration was used to calculate $K_F^{FAD}$ and $ΔG$ values for FAD binding (Table II). The T491V substitution was least disruptive, with approximately a 100-fold decrease in FAD binding affinity, indicating that the hydroxyl group of Thr491 contributes 2.8 kcal/mol of binding energy. In contrast, the R454E substitution produced a 25,000-fold decrease in FAD binding, corresponding to a destabilization of 6.0 kcal/mol. Effects of the Y456S and G488L

![Flavin Content](image)

**Protein**

Total flavin was determined by measurement of absorbance at 450 nm, and the FMN/FAD ratio was determined by the method of Faeder and Siegel (21). Values represent the mean ± S.D. for at least three separate protein preparations. □, FMN; ■, FAD. WT, wild type.
Reactions were carried out in 0.27 M potassium phosphate, pH 7.7, containing 65 μM cytochrome c and 50 μM NADPH. Reactions were carried out at 28 °C and initiated by the addition of protein. For Km measurements, concentrations of one substrate were held constant (65 μM cytochrome c for K_{FAD} and 50 μM NADPH for K_{NADPH}), and the concentration of the other substrate was varied. Values are expressed as mean ± S.D. (number of preparations).

| Protein | Specific activity | K_{FAD} | K_{NADPH} |
|---------|------------------|---------|-----------|
| Wild type | 57.4 ± 7.1 (4) | 16.3 ± 0.7 (4) | 62 ± 0.7 (4) |
| R454E | 0.17 ± 0.02 (3) | 16.6 ± 0.9 (3) | 4.1 ± 0.6 (3) |
| Y456S | 0.23 ± 0.02 (3) | 54.8 ± 1.3 (3) | 5.8 ± 0.8 (3) |
| G488L | 1.35 ± 0.16 (4) | 13.4 ± 1.6 (3) | 7.7 ± 0.4 (3) |
| T491V | 22.7 ± 3.7 (4) | 20.1 ± 2.5 (3) | 5.9 ± 0.4 (3) |
| C472T | 65.4 ± 21.3 (3) | 21.7 ± 2.4 (3) | 6.3 ± 0.6 (3) |
| W677Y | 36.0 ± 1.4 (4) | 14.3 ± 2.8 (3) | 2.8 ± 0.6 (3) |
| W677X | 1.7 ± 0.4 (3) | 8.5 ± 0.6 (3) | 1.5 ± 0.3 (4) |
| S678X | 62.5 ± 4.1 (3) | 19.9 (2) | 5.6 (2) |


d Data from Ref. 18.

Specific activity of wild-type enzyme (no FAD added).

For those mutants with elevated K_{FAD} and also measurable reductase activities (e.g. G488L and T491V), enzymic activity decreased rapidly over the course of the reaction, suggestive of FAD dissociation upon dilution into the assay buffer. Deviation from linearity is illustrated for T491V in Fig. 3. Linearity could be restored by inclusion of FAD in the assay buffer, confirming that the nonlinear behavior was due to FAD dissociation.

The rate of FAD dissociation from T491V was measured by preincubation of the wild-type and mutant proteins in 0.27 M potassium phosphate, pH 7.7, at 28 °C, prior to initiation of the cytochrome c reductase assay (Fig. 4). The wild-type enzyme exhibited a slow decrease in activity (Fig. 4A) with a rate constant (k_1 = 0.012 s^{-1}) consistent with FMN dissociation (12). In contrast, preincubation of the T491V protein in 0.27 M phosphate buffer yielded a biphasic curve with a rapid initial loss of activity (k_2 = 0.09 s^{-1}), followed by a continued decrease in activity (k_2 = 0.016 s^{-1}) (Fig. 4B). Inclusion of FAD, but not FMN, in the preincubation buffer prevented loss of activity. Dissociation was ionic strength-dependent, so that only a single slow (k_1 = 0.01 s^{-1}) phase was observed when T491V was preincubated in 0.01 M phosphate buffer (Fig. 4B).

In order to evaluate the relative flavin binding affinities of various mutants, the effect of urea on flavin release was measured (Fig. 5 and Table III). Dilution of wild-type reductase into 2 M urea produced a time-dependent increase in fluorescence, which corresponded to the release of approximately 55% of the total protein-bound flavin, consistent with the release of FMN but not FAD (12). In contrast, the magnitude of the fluorescence change for the T491V and Y456S mutants was significantly greater than 50% of the total fluorescence change, indicating dissociation of both FMN and FAD. The T491V mutant exhibited 77% of the total fluorescence change, with a rate constant 2.4 times that of wild-type. Although very little FAD was present in the Y456S mutant, this protein was reconstituted with FAD, and the rate of dissociation was measured.

Dilution of the reconstituted Y456S protein into urea produced 85% of the total fluorescence change, again consistent with dissociation of both FMN and FAD, with a rate constant 3.5 times the wild-type rate. Flavin dissociation from the G488L mutant was biphasic and was characterized by a rapid initial increase in fluorescence, with a rate 30 times that of wild type, followed by a slower increase.

**DISCUSSION**

Distinct binding regions for the prosthetic groups FMN and FAD were first identified through sequence homology (2), while subsequent crystallographic studies showed that these flavin binding sites existed as unique structural domains (15). Unlike FMN, FAD is tightly bound to the reductase, with a K_d < 1 nM (23), corresponding to a binding energy of >12 kcal/mol. Spe-
phases of dissociation, respectively, and samples for 5 min, centrifuging at 14,000 g for dissociation. Maximum fluorescence was determined by boiling the age of maximum fluorescence. Curves were fitted to the equations $F_t = F_0 + e^{-k_1 t}$ and $F_t = (F_0 + e^{-k_2 t})$, where $F_t$ equals fluorescence at time $t$, $F_0$ and $F_1$ represent the fluorescence associated with the first and second phases of dissociation, respectively, and $k_1$ and $k_2$ are the rate constants for dissociation. Maximum fluorescence was determined by boiling the samples for 5 min, centrifuging at 14,000 g for 5 min, and measuring the fluorescence of the supernatant. Values are expressed as percentages of maximum fluorescence. •, wild type; ○, Y456S; □, G488L; △, T491V.

**FIG. 5. Effect of urea on flavin release.** Wild-type (WT) and FAD-reconstituted mutant proteins (200 μg/ml) were contained in 50 mM Tris, pH 7.7, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 10% glycerol. Urea was added to a final concentration of 2 M, and fluorescence (excitation at 450 nm and emission at 535 nm) was monitored as a function of time. Curves were fitted to the equations $F_t = F_0 + e^{-k_1 t}$ and $F_t = (F_0 + e^{-k_2 t})$, where $F_t$ equals fluorescence at time $t$, $F_0$ and $F_1$ represent the fluorescence associated with the first and second phases of dissociation, respectively, and $k_1$ and $k_2$ are the rate constants for dissociation. Maximum fluorescence was determined by boiling the samples for 5 min, centrifuging at 14,000 g for 5 min, and measuring the fluorescence of the supernatant. Values are expressed as percentages of maximum fluorescence. •, wild type; ○, Y456S; □, G488L; △, T491V.

**TABLE III**

| Protein | $k_1$ | $k_2$ | Percentage of maximum* |
|---------|------|------|------------------------|
| Wild type | 0.10 ± 0.004 | 0.35 ± 0.01 | 55 |
| Y456S | 3.18 ± 0.6 | 3.18 ± 0.6 | 85 |
| G488L | 0.16 ± 0.02 | 0.24 ± 0.004 | 58 |
| T491V | 85 |

* Calculated as described in the legend to Fig. 4.

cific side chain interactions with FAD have been identified by crystallography, and the current study has evaluated the relative contributions of some of these interactions to flavin binding and the biochemical behavior of the reductase. Evidence has been presented both for the relative independence of the FMN- and FAD-binding domains as well as for interactions between the domains, which may be mediated through the interconnecting region (15). These results are consistent with earlier observations (9, 12, 13, 17–19).

The side chains of Arg454 and Tyr456 are major determinants of FAD binding, while hydrogen bonding interactions between the FAD pyrophosphate and the N-helix make smaller contributions. In terms of binding energy, replacement of the hydro-
Trp^{677} does facilitate NADP^{+} binding to NADPH-cytochrome P450 oxidoreductase (28).

Hydrogen bonding and van der Waals interactions between FAD and amino acid residues in the FAD binding pocket of the reductase serve to regulate both flavin binding and reactivity. The present study has utilized site-directed mutagenesis to assess the functional significance of FAD binding site residues identified by x-ray crystallography. Residues 488–491 and Arg^{454} function primarily in FAD binding, while Tyr^{456} has dual FAD-binding and catalytic functions and Trp^{677} regulates pyridine nucleotide binding and catalysis. Finally, the interaction between the two flavins of NADPH-cytochrome P450 reductase has also been a subject of much interest. Our studies suggest that, although the FMN and FAD domains are competent in binding FMN and FAD independently, alterations in one or the other domain do have an impact on the integrity of the other. Studies to elucidate the mechanism of these interdomain interactions are under way.

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REFERENCES

1. Porter, T. D. & Kasper, C. B. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 973–977
2. Porter, T. D. & Kasper, C. B. (1986) Biochemistry 25, 1682–1687
3. Porter, T. D. (1991) Trends Biochem. Sci. 16, 154–158
4. Shen, A. L & Kasper, C. B. (1993) in Handbook of Experimental Pharmacology (Schenkman, J. B., & Greim, H., eds) Vol. 105, pp. 35–59, Springer-Verlag, Berlin
5. Ostrowski, J., Barber, M. J., Rueger, D. C., Miller, B. R., Siegel, L. M. & Kredich, N. M. (1989) J. Biol. Chem. 264, 15796–15808
6. Ruettinger, R. T., Wen, L. P. & Pulco, A. J. (1989) J. Biol. Chem. 264, 10987–10995
7. Bredt, D. S., Hwang, P. M., Glatt, C. E., Lowenstein, C., Reed, R. R. & Snyder, S. H. (1991) Nature 351, 714–718
8. LeClerq, D., Wilson, A., Damas, R., Gafuik, S., Song, D., Watkins, D., Heng, H. H. Q., Rommens, J. M., Scherer, S. W., Rosenblatt, D. S. & Gravel, R. A. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 3659–3664
9. Shen, A. L., Porter, T. D., Wilson, T. E. & Kasper, C. B. (1989) J. Biol. Chem. 264, 7584–7589
10. Sem, D. S. & Kasper, C. B. (1993) Biochemistry 32, 11548–11558
11. Shen, A. L. & Kasper, C. B. (1986) J. Biol. Chem. 270, 27475–27480
12. Hodgson, A. V. & Strobel, H. W. (1996) Arch. Biochem. Biophys. 325, 99–106
13. Smith, G. C., Tew, D. G. & Wolf, C. R. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 8710–8714
14. Black, S. D. & Coon, M. J. (1982) J. Biol. Chem. 257, 5929–5938
15. Wang, M., Roberts, D. L., Paschke, R., Shea, T. M., Masters, B. S. & Kim, J.-J. P. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 8411–8416
16. Barsukov, I., Modi, S., Lian, L.-Y., Sze, K. H., Paine, M. J. I., Wolf, C. R. & Roberts, G. C. R. (1997) J. Biol. Chem. 270, 63–75
17. Vermilion, J. L. & Coon, M. J. (1978) J. Biol. Chem. 253, 8812–8819
18. Kurzban, G. P. & Strobel, H. W. (1986) J. Biol. Chem. 261, 7824–7830
19. Narayansswami, R., Hurwitz, P. M. & Masters, B. S. (1995) Arch. Biochem. Biophys. 316, 267–274
20. Smith, P. K. (1985) Anal. Biochem. 150, 76–85
21. Faeder, E. J. & Siegel, L. M. (1973) Anal. Biochem. 53, 332–336
22. Calceterra, N. B., Pico, G. A., Orellano, E. G., Ottado, J., Carrillo, N. & Ceccarelli, E. A. (1995) Biochemistry 34, 12842–12848
23. Kurzban, G. P., Howarth, J., Palmer, G. & Strobel, H. W. (1990) J. Biol. Chem. 265, 12272–12279
24. Karplus, P. A., Daniels, M. J. & Herriott, J. R. (1991) Science 251, 60–66
25. Sem, D. S. & Kasper, C. B. (1994) Biochemistry 33, 12012–12021
26. Dwyer, T. M., Morti, S., Kemter, K., Bacher, A., Faug, A. & Frerman, P. (1999) Biochemistry 38, 9735–9745
27. Deng, Z., Aliverti, A., Zanetti, G., Arakaki, A. K., Ottado, J., Orellano, E. G., Calceterra, N. B., Ceccarelli, E. A., Carrillo, N. and Karplus, P. A. (1999) Nat. Struct. Biol. 6, 847–853
28. Hubbard, P. A., Paschke, B., Kim, J.-J. P., Shen, A. L., and Kasper, C. B. (1999) in Flavins and Flavoproteins (Ghisla, S., Kroneck, P., Marcheroux, P., and Sund, H., eds) pp. 159–162, Agency for Scientific Publication, Berlin
29. Guex, N., and Peitsch, M. C. (1997) Protein Data Bank Quarterly Newsletter 77, 7.