Biochemical and Biophysical Research Communications **254,** 83–87 (1999)
Article ID bbrc.1998.9889, available online at http://www.idealibrary.com on IDEAL®

Some Properties of Mitochondrial Adrenodoxin Associated with Its Nonconventional Electron Donor Function toward Rabbit Liver Microsomal Cytochrome P450 2B4

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Received November 5, 1998

Mitochondrial adrenodoxin (Adx) was found to cross-react with microsomal cytochrome P450 2B4 (CYP2B4) as the terminal electron acceptor. When compared with NADPH-cytochrome P450 reductase (P450R), the natural redox partner of CYP2B4, Adx was less efficient both in transferring the first electron and in coupling the system. The ferredoxin yielded an unusual reverse type I spectral change with low-spin CYP2B4, which underwent transformation to a typical type I optical perturbation upon deletion of the signal anchor sequence (Δ2-27) of the hemoprotein. Truncation of CYP2B4 slightly fostered electron transfer from Adx, but was deleterious to reduction of the engineered isozyme by P450R. Addition of manganese-substituted cytochrome b_5, which failed to serve as an electron donor to CYP2B4, augmented the amount of hemoprotein existing in form of a low-spin complex with Adx and affected the ferredoxin-dependent reduction kinetics through causing a proportional rise in both K_m and V_{max}. Conservative replacement of Asp-76 with glutamate in the Adx molecule was associated with a drastic drop in reductive efficiency toward CYP2B4, while spectral binding of the mutant to the hemoprotein was marginally changed. The results support the concept of an evolutionary relationship between the various cytochrome P450 forms as regards the conservation of surface regions participating in contacts with heterologous donor proteins.

Cytochrome P450 (P450 or CYP; EC 1.14.14.1) enzymes represent a group of structurally related hemoproteins involved in the oxygenation of a variety of exo- and endobiotic compounds (1). P450 monooxygenases have been classified into two categories based on their electron-supplying redox partners. Thus, in one class of cytochromes P450, comprising mitochondrial and bacterial isoforms, an FAD-containing enzyme, ferredoxin reductase (EC 1.18.1.2), and an iron-sulfur ferredoxin of the Fe_2S_2 type serve for bridging reducing equivalents from NAD(P)H to the terminal acceptor (2). In the other class, which includes microsomal P450 isozymes, NADPH-P450 reductase (P450R; EC 1.6.2.4) has been recognized to be the chemically competent electron carrier that contains one molecule each of FAD and FMN (3).

Initial observations failed to substantiate cross-reactivity of redox components purified from pro- and eukaryotic systems (4), suggesting high specificity for the individual donor/acceptor couples. However, subsequent studies revealed that ferredoxins (flavodoxins) isolated from microbial and plant sources, respectively, were capable of supporting catalytic activities when reconstituted with a diversity of microsomal P450 species (5-8). Similarly, an N-terminal-modified rat mitochondrial P450c27, targeted to the yeast microsomal compartment, could be reconstituted with yeast microsomal P450R (9).

The interchangeability of microsomal P450R with mitochondrial ferredoxins in reductive processes involving microsomal cytochromes P450 lacks appreciable documentation. Here, we delineate similarities and differences between the bovine adrenodoxin (Adx) system and P450R as electron donors to rabbit liver microsomal CYP2B4.

MATERIALS AND METHODS

Materials. NADPH, glucose 6-phosphate, glucose-6-phosphate dehydrogenase (EC 1.1.1.49), glucose oxidase (EC 1.1.3.4) and catalase (EC 1.11.1.6) were obtained from Boehringer (Mannheim, Ger-

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Abbreviations used: P450 or CYP, cytochrome P450; Mn-b_5, manganese-substituted cytochrome b_5; Adx, adrenodoxin; P450R, NADPH-cytochrome P450 reductase; AdR, NADPH-adrenodoxin reductase.

0006-291X/99 $30.00

many). Dilauroyl L-α-phosphatidylcholine and hexobarbital were purchased from Sigma (Deisenhofen, Germany). All other reagents were of the highest purity commercially available.

Protein expression and purification. Truncated CYP2B4 lacking amino acid residues 2-27, CYP2B4 (Δ2-27), was expressed fused to glutathione *S*-transferase in *E. coli* host strain JM109 (Stratagene, Heidelberg, Germany), liberated from the transferase moiety by thrombin treatment, and purified to a specific content of 15 nmol/mg protein exactly as described previously (10). Full-length CYP2B4 was purified to apparent electrophoretic homogeneity from hepatic microsomes of male New Zealand White rabbits pretreated with phenobarbital (50 mg/kg) for 7 consecutive days (11); the specific hemoprotein content averaged 17 nmol/mg protein. The concentration of the native and recombinant pigment was determined as indicated by Omura and Sato (12) using an absorption coefficient of 91 mM^{-1} cm^{-1}.

Manganese-substituted cytochrome b_5 (Mn-b_5) was prepared by modification of rabbit liver microsomal b_5 as detailed elsewhere (13) and quantified using an absorption coefficient for the reduced protein of 75 mM^{-1} cm^{-1} at 432 nm.

Wild type adrenodoxin and its site-directed mutant were expressed in *E. coli* and purified as reported previously (14). The A_{414}/A_{276} ratio of the preparations was 0.9. The concentration of the recombinant proteins was determined using an absorption coefficient of 9.8 mM^{-1} cm^{-1} at 414 nm (15).

NADPH-adrenodoxin reductase (AdR) and P450R, isolated by establishes procedures from bovine adrenocortical mitochondria (16) and rabbit liver microsomes (11), were measured on the basis of their absorbance at 450 and 456 nm, respectively, using absorption coefficients of 11.3 and 21.4 mM^{-1} cm^{-1} (17,18).

Spectral measurements. Binding of Adx to P450 was studied using a set of matched tandem cuvettes. The final assay mixtures consisted of 2 μM native or truncated CYP2B4, 4 μM Adx protein, and 48 μM dilauroyl phosphatidylcholine in 100 mM sodium phosphate, pH 7.4, containing 20% glycerol and 0.04% Emulgen 911. For some measurements, the incubation media were supplemented with 4 μM Mn-b_5. Optical complex formation was followed at 25°C by scanning difference spectra in the region from 370 to 450 nm with a Shimadzu UV-265 FW spectrophotometer; optical pathlength was 1.0 cm.

Enzyme assays. The kinetics of NADPH-supported reduction of ferric P450 were assessed at 25°C in anaerobic reaction mixtures comprising 1 to 2 μM native or truncated CYP2B4, varying amounts of P450R (or Adx protein plus 0.3 μM AdR), 48 μM dilauroyl phosphatidylcholine (sonicated until clarification was observed), 1 mM hexobarbital, 100 mM glucose, glucose oxidase (400 μg/ml), and catalase (75 μg/ml) in 100 mM sodium phosphate, pH 7.4; some assays were carried out in the presence of 4 μM Mn-b_5. The systems were preincubated at room temperature for 15 min to allow efficient association of the redox partners. Subsequently, the samples were gassed for 5 min with CO. Reactions were initiated by the rapid addition, in a plunger cuvette, of NADPH to yield a final concentration of 1 mM. Absorbance changes at 450 nm relative to 500 nm were monitored with an Aminco DW-2 spectrophotometer operated in the dual-wavelength mode.

NADPH oxidase activity was measured at 25°C in aerobic media composed of 1 μM CYP2B4, 0.6 μM P450R (or 7μM Adx plus 0.3 μM AdR), 48 μM dilauroyl phosphatidylcholine, 2 mM hexobarbital, and 50 μM NADPH in 100 mM sodium phosphate, pH 7.4. Disappearance of the reduced cofactor was followed by the decrease in absorbance at 340 nm using an absorption coefficient of 6.22 mM^{-1} cm^{-1} (19). For determining H_2O_2 production, the above mixtures were fortified with NADPH-regenerating system consisting of 10 mM glucose 6-phosphate, 6 mM $MgCl_2$, and glucose-6-phosphate dehydrogenase (5μg/ml) and incubated at room temperature for 15 min. Peroxide was quantified by the ferrithiocyanate method (20).

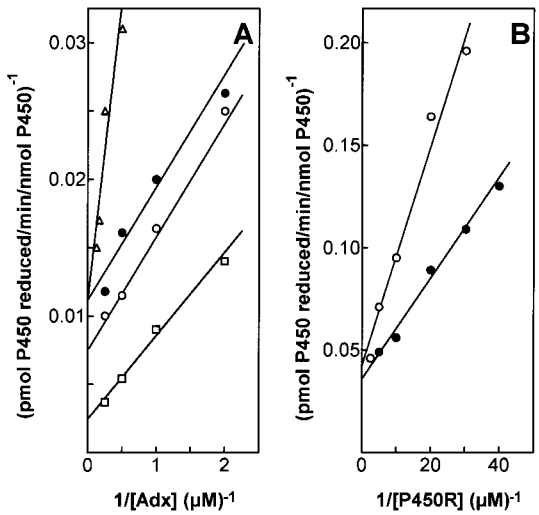

FIG. 1. Initial-velocity patterns of NADPH-sustained CYP2B4 reduction by Adx and P450R. The initial rates of cofactor-driven formation of the ferrous-carbonyl adduct of P450 were assessed at 25°C by monitoring the absorbance change at 450 nm, the basic reaction mixtures containing ferric hemoprotein, phospholipid, hexobarbital and either Adx/AdR (A) or P450R (B) as the electron donors. The particular redox systems studied were as follows. Panel A: ●, Adx/CYP2B4; ○, Adx/CYP2B4/Mn-b_5; □, Adx/CYP2B4(Δ2-27); △, Adx(D76E)/ CYP2B4. Panel B: ●, P450R/CYP2B4; ○, P450R/ CYP2B4(Δ2-27). The lines were obtained by non-linear regression analysis using the Corel QuattroPro 7. 0 software. The points represent the means of two to three experiments.

RESULTS AND DISCUSSION

The main concern of the present study was to compare Adx and P450R with respect to their capacity to donate electrons to CYP2B4. To this end, NADPH-supported, hexobarbital-stimulated reduction of the ferric hemoprotein was assessed in reconstituted micellar systems containing either P450R or Adx/AdR as the redox partner. As regards the latter chain, a saturating concentration of AdR, corresponding to about 50-times the apparent K_m value for the reductase (21), was applied throughout our experiments. Since flavin-to-iron/sulfur electron transfer was not rate limiting under these conditions (14), reduction activity toward CYP2B4 became entirely Adx-dependent. Measurements were conducted under strictly anaerobic conditions to avoid autoxidation of the terminal acceptor. Hence, observed velocities of accumulation of ferrous CYP2B4 primarily reflected rates of electron flow. The kinetic data derived from such assays are plotted in Figure 1 and are summarized in Table 1. As can be seen, both P450R and Adx were active, but the apparent K_m value for the former donor was found to be 12-times lower as compared with that for the ferredoxin, while the reductive capacity (V_{max} value) of the Adx-linked system was about 3-times that of the P450R-sustained one. From these data, the efficiency of electron transfer (V_{max}/K_m) in the Adx-dependent

TABLE 1

Initial-Velocity Kinetic Constants for the Reduction of Ferric CYP2B4 by Adx and P450R

	Cytochrome P450 reduction		
Redox system studied	K_m (μM)	V_{max} (pmol/min/ nmol P450)	V_{max}/K_m
Adx/CYP2B4	0.73 ± 0.05	91.1 ± 6.5	124.8 ± 8.9
Adx/CYP2B4/Mn-b_5	1.16 ± 0.08	132.6 ± 9.4	114.3 ± 8.1
Adx/CYP2B4(Δ2-27)	2.32 ± 0.14	395.2 ± 24.5	170.3 ± 10.4
Adx(D76E)/CYP2B4	3.87 ± 0.16	91.6 ± 3.8	23.7 ± 0.9
P450R/CYP2B4	0.06 ± 0.01	27.5 ± 0.9	458.3 ± 15.6
P450R/CYP2B4(Δ2-27)	0.12 ± 0.01	23.1 ± 1.5	192.5 ± 12.2

Note. The apparent K_m and V_{max} values, reflecting the reactivities and reductive capacities of Adx protein and P450R toward CYP2B4, were taken from the double-reciprocal initial-velocity plots presented in Figure 1. Calculations were carried out by non-linear regression analysis using the Corel QuattroPro 7.0 software. The data are the means ± SEM of two to three experiments.

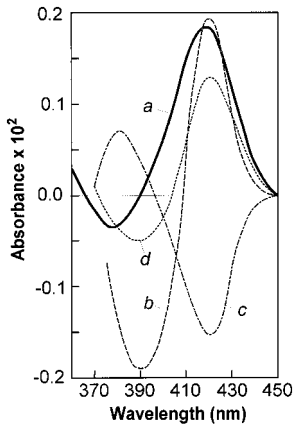

FIG. 2. Spectral binding of Adx to CYP2B4. Difference spectra were obtained by reacting 2 μM native (*a*,*b*,*d*) or truncated (*c*) CYP2B4 with 4 μM wild type (*a*,*b*,*c*) or mutated (*d*) Adx reconstituted into phospholipid in the absence (*a*,*c*,*d*) or presence (*b*) of 4 μM Mn-b_5.

route could be calculated to be 27% that in the P450R-promoted pathway.

In another set of experiments, electron transfer to CYP2B4, as judged by NADPH oxidation in the presence of hexobarbital, was investigated under aerobic conditions along with cofactor-driven H_2O_2 formation at saturating level (~$10K_m$) of P450R and Adx, respectively. As shown in Table 2, the proportion of peroxide formed to NADPH utilized with barbiturate present was strongly dependent on the donor operative, oxyferrous CYP2B4 being more readily autoxidizable when Adx was the supplier of reducing equivalents. This was interpreted to mean that Adx was less efficient than P450R in coupling the system through donation of the second electron to the oxygenated hemoprotein. Alternatively, binding of Adx to CYP2B4 might have destabilized the protonated iron-peroxo intermediate to release peroxide. On the basis of these findings and that of a previous report, demonstrating that CYP2B4 can undergo reduction by bacterial redoxins (5), we concluded that CYP2B4 lacked specificity for P450R as the electron donor. Nevertheless, the flavoprotein reductase appeared to be the preferred redox partner for this P450 isozyme.

TABLE 2

Rates of Aerobic NADPH Consumption and H_2O_2 Production by CYP2B4 Reconstituted with Adx/AdR or P450R in the Presence of Hexobarbital

	Activity (nmol/min per nmol CYP2B4)		
Donor system	NADPH oxidation (a)	H_2O_2 production (b)	(b)/(a)
Adx/AdR	2.15 ± 0.21	1.57 ± 0.07	0.73 ± 0.04
P450R	2.28 ± 0.19	0.57 ± 0.02	0.25 ± 0.01

Note. Assays were performed at 25°C as detailed under "Materials and Methods" by following cofactor utilization at 340 nm and peroxide-linked formation of ferrithiocyanate at 480 nm. The data represent the means ± SEM of two to three experiments.

In order to better understand the aberrant kinetic behaviour between Adx and P450R, studies were designed to analyze peculiarities in the interaction of the two donors with CYP2B4. Thus, the discrepancy in the affinities of Adx and P450R for the hemoprotein (Table 1) prompted us to examine the nature of ferredoxin binding to CYP2B4 more closely using difference spectroscopy as a probe for complex formation. Most strikingly, Adx produced a reverse type I spectral change, also termed modified type II, characterized by a Soret band at 420 nm and a trough at 380 nm when reacted with CYP2B4 in the presence of phospholipid (Figure 2, curve *a*), while P450R has been previously demonstrated to generate a typical type I optical perturbation with this P450 form (22). The unusual Adx-induced spectral species could be ruled out to arise from the fact that excess dilauroyl phosphatidylcholine, which itself elicits a type I optical shift with CYP2B4 (23), might have displaced the ferredoxin from some type I binding site to unmask a type II component hidden in the difference spectrum: omission of the phospholipid from the assay media attenuated the amplitude of the reverse type I spectral change by 47%, but failed to abolish its basic features (data not shown). Unlike the mitochondrial CYP11A1 (24), low-spin CYP2B4 thus gave a difference spectrum with Adx. The question arose as to whether the NH_2-terminal, membrane insertable signal anchor sequence of CYP2B4 might have played some role in Adx binding. Indeed, association of the ferredoxin with cytochrome lacking amino acid residues 2-27 resulted in transition of the reverse to a regular type I high-spin complex exhibiting a maxi-

mum at 380 nm and a minimum at 420 nm (Figure 2, curve *c*). Thus, the structural integrity of the hydrophobic tail portion of CYP2B4 was recognized to be intrumental, by direct or/and indirect means, to the specific mode of Adx anchoring. The observed low-to-high spin switch in complexing of the ferredoxin was obviously beneficial to electron transfer, as there was a significant ($p < 0.05$) increase by 36% in the V_{max}/K_m value in the presence of hexobarbital (Figure 1A and Table 1). In contrast to these findings, previous and present data from our laboratory show that deletion of residues 2-27 in the CYP2B4 molecule failed to affect the mode of P450R binding to the engineered enzyme (22) and caused a marked drop by 58% in reductive efficiency when P450R was the donor of reducing equivalents (Figure 1B and Table 1). It should be pointed out that the impact of CYP2B4 truncation on the kinetics of Adx- and P450R-directed cytochrome reduction was unlikely to arise from aberrant partitioning of the modified hemoprotein between the lipid and aqueous phases of the assay media to alter the statistical probability of the redox partners to encounter one another, since we have recently shown that loss of residues 2-27 in the NH_2-terminal region of CYP2B4 did not restrict access of the mutant enzyme to micellar phospholipid (22).

Attention was drawn to the fact that b_5 had been previously reported to act on certain P450 isoforms such as to modify catalytic activities via mechanism(s) not accounted for by its role as an electron carrier (13,25). Therefore, we have used manganese-substituted b_5 in an attempt to elucidate a possible effector function of the pigment when reconstituted under anaerobic conditions with CYP2B4 and the Adx-dependent redox chain in the presence of NADPH. It has to be emphasized that we were unable to detect measurable NADPH-driven reduction of the oxidized Mn-b_5 by Adx/AdR (data not shown). This finding was analogous to the failure of the modified polypeptide to accept electrons from P450R (13), excluding donor properties toward P450. However, binding of the manganese-substituted derivative to CYP2B4 was assumed to proceed in a way comparable with that of b_5, since available data from resonance Raman spectroscopy indicated a structure of the hexacoordinated Mn-b_5 quite similar to that of the native congener (26).

Inclusion of Mn-b_5 in the assay mixtures increased the amount of CYP2B4 existing in form of a low-spin adduct with Adx (Figure 2, curve *b*) and provoked a shift of the kinetic tracings for Adx-mediated electron transfer to the ferric hemoprotein parallel to those obtained in the absence of the modifier (Figure 1A). This was associated with a significant (p_m and V_{max}, so that reductive efficiency remained unchanged (Table 1). This was taken to indicate that docking of Mn-b_5 to CYP2B4 might have brought about a conformational rearrangement(s) on the surface of the isozyme serving to directly enhance k_{cat} for reduction, the observed decrease in affinity of Adx most likely resulting from an important numerical contribution of k_{cat} to the value of K_m (27). On the other hand, previous studies by our laboratory failed to substantiate any measurable influence of Mn-b_5 on the kinetics of NADPH-sustained CYP2B4 reduction when catalyzed by the P450R component (28).

Further information on the mode of Adx binding to CYP2B4 came from experiments with ferredoxin, in which aspartate located at position 76 in the highly conserved acidic domain had been replaced with glutamate via site-directed mutagenesis; such a substitution was of interest, since Asp-76 has been reported to be critical for the binding of both AdR and CYP11A1 (14, 29). The recombinant Adx (D76E) protein exhibited unchanged redox properties and CD as well as EPR spectra characteristic of regular assembly of the iron-sulfur cluster, while affinity for AdR was markedly diminished (14). The latter was compensated for by the large excess of AdR used throughout our measurements (see above).

The conservative exchange of the polar residue in the Adx molecule maintained the negative charge at position 76, but was thought to affect the precise steric positioning of the carboxyl moiety such as to impede its short-range interactions. In fact, the mutant D76E exhibited a 5.3-fold increase in the K_m value with undisturbed reductive potency (V_{max} value) toward CYP2B4, so that V_{max}/K_m levelled down to a value 19% that for the wild type (Figure 1A and Table 1). When CYP2B4 was reacted with a 2-fold molar excess of engineered ferredoxin, a stoichiometry at which cytochrome reduction was decelerated by 51% (cf. Figure 1A), the magnitude of the resultant reverse type I optical change was decreased by some 18% relative to the Adx-induced spectrum (Figure 2, curve *d*). Unless one assumes that the latter was a token of unspecific interaction of the redox components, the above data would have suggested that hampered non-productive binding of the mutated ferredoxin to CYP2B4, possibly affecting precollision spacial orientation of the partners, did not constitute the overriding mechanism responsible for the pronounced disruption of electron flow. Rather, defective alignment, due to loss of a fixed geometry, of site(s) involved in the immediate transfer of reducing equivalents might have been of vital importance (29).

Collectively, we have furnished experimental evidence of the ability of mitochondrial Adx to cross-react with microsomal CYP2B4 as the terminal electron acceptor. This finding supports and extends previous work (5-9) on a possible evolutionary relationship between the diverse P450 forms as regards the conservation of surface regions participating in interactions with heterologous, nonconventional donor proteins. With some P450 isozymes, such sites appear to be located remote from each other, as suggested by the

distinct spectral complexes generated with CYP2B4 and either Adx or P450R and the kinetic response of the ferredoxin to the presence of Mn-b_5. With other types of P450, contact regions might be accomodated close to each other or overlap. Thus, there seems to exist competition of putidaredoxin and microsomal b_5 for a common domain on microbial CYP101 (30). Similarly, b_5 has been reported to displace Adx from some mutually exclusive patch on the surface of mitochondrial CYP11A1 (31). A more detailed molecular delineation of specific protein-protein associations across the P450 family will be the subject of future studies.

ACKNOWLEDGMENTS

This work was supported by grants Hl 1/15-1 and Be 1343/1-3 from the Deutsche Forschungsgemeinschaft. The authors are indepted to Cortina Keiling for competent technical assistance.

REFERENCES

1. Porter, T. D., and Coon, M. J. (1991) *J. Biol. Chem.* **266,** 13469–13472.
2. Mueller, E. J., Loida, P. J., and Sligar, S. G. (1995) *in* Cytochrome P450. Structure, Mechanism, and Biochemistry (Ortiz de Montellano, P. R., Ed.), pp. 83–124, Plenum Press, New York.
3. Lu, A. Y. H., and Coon, M. J. (1968) *J. Biol. Chem.* **243,** 1331–1332.
4. Dus, K. (1975) *Adv. Exp. Med. Biol.* **58,** 287–309.
5. Bernhardt, R., and Gunsalus, I. C. (1992) *Biochem. Biophys. Res. Commun.* **187,** 310–317.
6. Yamazaki, H., Ueng, Y. F., Shimada, T., and Guengerich, F. P. (1995) *Biochemistry* **34,** 8380–8389.
7. Dong, M. S., Yamazaki, H., Guo, Z., and Guengerich, F. P. (1996) *Arch. Biochem. Biophys.* **327,** 11-19.
8. Jenkins, C. M., and Waterman, M. R. (1998) *Biochemistry* **37,** 6106–6113.
9. Sakaki, T., Kominami, S., Hayashi, K., Akiyoshi-Shibata, M., and Yabusaki, Y. (1996) *J. Biol. Chem.* **271,** 26209–26213.
10. Pernecky, S. J., Olken, N. M., Bestervelt, L. L., and Coon, M. J. (1995) *Arch. Biochem. Biophys.* **318,** 446–456.
11. Hlavica, P., and Hülsmann, S. (1979) *Biochem. J.* **182,** 109–116.
12. Omura, T., and Sato, R. (1964) *J. Biol. Chem.* **239,** 1379–2385.
13. Morgan, E. T., and Coon, M. J. (1984) *Drug. Metab. Dispos.* **12,** 358–364.
14. Beckert, V., and Bernhardt, R. (1997) *J. Biol. Chem.* **272,** 4883–4888.
15. Kimura, T. (1968) *in* Structure and Bonding (Jorgensen, C. K., Neilands, J. B., Nyholm, R. S., Reinen, D., and Williams, R. J. P., Eds.), Vol. 5, pp. 1–40, Springer-Verlag, Berlin.
16. Akhrem, A. A., Lapko, V. N., Lapko, A. G., Shkumatov, V. M., and Chashchin, V. L., (1979) *Acta Biol. Med. Ger.* **38,** 257–274.
17. Hiwatashi, A., Ichikawa, Y., Maruya, N., Yamano, T., and Aki, K. (1976) *Biochemistry* **15,** 3082–3090.
18. French, J. S., Guengerich, F. P., and Coon, M. J. (1980) *J. Biol. Chem.* **255,** 4112–4119.
19. Horecker, B. L., and Kornberg, A. (1948) *J. Biol. Chem.* **175,** 385–390.
20. Hildebrandt, A. G., Roots, I., Tjoe, M., and Heinemeyer, G. (1978) *Methods Enzymol.* **52,** 342–350.
21. Uhlmann, H., and Bernhardt, R. (1995) *J. Biol. Chem.* **270,** 29959–29966.
22. Lehnerer, M., Schulze, J., Pernecky, S. J., Lewis, D. F. V., Eulitz, M., and Hlavica, P. (1998) *J. Biochem.* **124,** 396–403.
23. Chiang, Y. L., and Coon, M. J. (1979) *Arch. Biochem. Biophys.* **195,** 178–187.
24. Kido, T., and Kimura, T. (1979) *J. Biol. Chem.* **254,** 11806–11815.
25. Auchus, R. J., Lee, T. C., and Miller, W. L. (1998) *J. Biol. Chem.* **273,** 3158–3165.
26. Gruenke, L. D., Sun, J., Loehr, T. M., and Waskell, L. (1997) *Biochemistry* **36,** 7114–7125.
27. Segel, I. H. (1975) Enzyme Kinetics, John Wiley & Sons, New York.
28. Golly, I., Hlavica, P., and Schartau, W. (1988) *Arch. Biochem. Biophys.* **260,** 232–240.
29. Coghlan, V. M., and Vickery, L. E. (1992) *J. Biol. Chem.* **267,** 8932–8935.
30. Stayton, P. S., Poulos, T. L., and Sligar, S. G. (1989) *Biochemistry* **28,** 8201–8205.
31. Usanov, S. A., and Chashchin, V. L. (1991) *FEBS Lett.* **278,** 279–282.