Epoxidation of Unsaturated Fatty Acids by a Soluble Cytochrome P-450-dependent System from Bacillus megaterium*

(Received for publication, December 23, 1980, and in revised form, March 9, 1981)

Richard T. Ruettinger and Armand J. Fulco

From the Department of Biological Chemistry, UCLA School of Medicine and the Laboratory of Biomedical and Environmental Sciences, University of California, Los Angeles, California 90024

In previous publications from this laboratory we have described a soluble, partially purified cytochrome P-450-dependent monoxygenase complex that, in the presence of NADPH and O₂, catalyzes the monohydroxylation of long chain fatty acids, alcohols, and amides at the ω − 1, ω − 2, and ω − 3 positions. We have now found that this preparation catalyzes the epoxidation as well as the hydroxylation of palmitoleic acid and a variety of other monounsaturated fatty acids. The experimental results reported here strongly support the concept that both hydroxylation and epoxidation are catalyzed by an identical cytochrome P-450 complex utilizing the same active and binding sites. Furthermore, for saturating levels of these substrates, the rate-limiting step in oxygenation does not appear to involve substrate structure. Thus, although the position and geometry of the double bond may dramatically affect the rate of epoxidation relative to hydroxylation, the combined rate of substrate oxygenation is essentially a constant independent of this ratio. Finally, we propose and present evidence for an enzyme-substrate binding model that involves polar binding of the carboxyl terminus and strong hydrophobic binding and sequestering of the terminal methyl group of the fatty acid. The three methylene carbons adjacent to the methyl group are positioned in a set geometry around the active site but the midchain region of a monounsaturated fatty acid is relatively free to interact or bind loosely with the enzyme surface in a variety of conformations. Depending on fatty acid structure, one or more of these conformations can bring the unsaturated center close enough to the active site to permit epoxidation of the double bond.

The metabolism of aromatic and aliphatic epoxides in animal tissues has received increasing notice in the past decade, especially in view of the potential role of certain of these reactive molecules as toxic or carcinogenic agents. Paradoxically, detoxification and elimination of a variety of drugs and xenobiotic compounds also involve enzymatic epoxidation (Oesch, 1973; Sims and Grover, 1974) as do a number of essential metabolic processes, such as cholesterol biosynthesis (Ono and Bloc, 1975), steroid transformations (Watabe and Sawahata, 1979; Watabe et al., 1979), and vitamin K-dependent thrombin production (Willingham and Matschner, 1974).

* This work was supported by National Institutes of Health Research Grant GM-23913 and by Contract DE-AM03-76-SF00012 between the Department of Energy and the University of California.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Although enzyme-catalyzed epoxidation of unsaturated fatty acids has not, to our knowledge, been studied in mammalian systems, it has been demonstrated in plants. The accumulation of high levels of epoxy fatty acids in certain seed oils and fungal spores (Sessa et al., 1977; Weete and Kelly, 1977) is presumably the result of enzymatic epoxidation. Croteau and Kolastrakody (1975) have shown that a soluble p-galactosidase fraction from spinach could catalyze the epoxidation of the double bond of 18-hydroxoleate. Since this reaction required NADPH and O₂ and showed light-reversible inhibition by CO, it was assumed that a cytochrome P-450 system was involved. The epoxidation of lipophilic substances in bacteria has received less attention than the analogous processes in eukaryotes but several examples are known. The short chain fatty acid and alkane ω-oxidation systems isolated from Pseudomonas oleovorans by Coon and his co-workers (Griffith et al., 1978) can also epoxidize the analogous unsaturated derivatives (May and Abbott, 1972, 1973). This mixed-function oxygenase, which is isolated as an aggregate, requires phospholipid for activity and utilizes a non-heme iron prosthetic group (Ruettinger et al., 1974, 1977). In Bacillus megaterium ATCC 14581, we have found a soluble, cytochrome P-450-dependent monoxygenase complex (molecular weight between 135,000 and 150,000) that, in the presence of NADPH and O₂, catalyzes the monohydroxylation of long chain fatty acids, alcohols, and amides at the ω − 1, ω − 2, and ω − 3 positions (Miura and Fulco, 1974, 1975; Hare and Fulco, 1975; Ho and Fulco, 1976; Matson et al., 1977). It should be noted that this system is quite distinct from the cytochrome P-450 system isolated from another strain of B. megaterium and characterized by Berg and his co-workers (1975, 1976, 1979). The P-450 system shows a weak hydroxylating activity for 3-oxo-Δ⁴-steroids but apparently does not utilize fatty acids as substrates nor does it appear to catalyze epoxidations. On the other hand, when our partially purified preparation from B. megaterium 14581 was tested with palmitoleic acid, in addition to the expected isomeric mixture of monohydroxy-palmitoleates, 9,10-epoxy-palmitate and 9,10-dihydroxy-palmitate were also identified as reaction products (Buchanan and Fulco, 1978). We later demonstrated that the dihydroxy derivative was formed from epoxy-palmitate by a soluble epoxide hydrolase. Careful gel filtration chromatography resulted in complete separation of this hydrolyase from monoxygenase activity but did not resolve epoxidation and hydroxylation activities (Michaels et al., 1980). We now wish to report the results of experiments that describe the epoxidation reaction and explore its relationship to the cytochrome P-450-catalyzed fatty acid hydroxylation system.

EXPERIMENTAL PROCEDURES

Materials—NADPH, NADH, Sephadex G-200, and Sephacryl S-300 were obtained from Sigma. All labeled fatty acids, except for...
Patty Acid Epoxidation by a Cytochrome P-450 System

those listed below, were purchased from Rosechem Products. [1-\(^{14}\)C]Palmitic acid was purchased from New England Nuclear Corp. [1-\(^{14}\)C]cis-10-Hexadecenoic acid (98% radioactivity) was obtained from Bacillus licheniformis ATCC 9259 that had been grown at 36 °C in the presence of [1-\(^{14}\)C]palmitate. The isolation and purification of this substrate has been described in detail (Fulco, 1970). [1-\(^{14}\)C]cis-10-octadecenoic acid (96% radioactivity) and [1-\(^{14}\)C]cis-5-tetradecenoic acid (99% radioactivity) were synthesized in toto by the procedure of Fulco (1970). The cis and trans isomers of [1-\(^{14}\)C]epoxypalmitate (96% radioactivity) were prepared by treatment of labeled palmitoleate and palmitolactate respectively with peracetic acid as described previously (Michaels et al., 1980). Unlabeled 12-
hydroxysearc acid was purchased from J. C. Baker Co. All other nonradioactive substrates were purchased from Applied Science. Enzyme Preparation—The conditions for the growth and harvest-
ing of B. megaterium 14581 have been described previously (Miura and Fulco 1974). Extracts containing the monoxygenase activity were prepared by treating cells suspended in 0.1 M potassium phosphate buffer, pH 7.0, by a combination high pressure nitrogen bomb-sion technique as described by Miura and Fulco (1974). The resulting preparation was centrifuged at 27,000 × g for 30 min to remove debris and the clear supernatant was then subjected to (NH₄)₂SO₄ fractionation. The protein precipitating at 40-55% saturation contained most of the P-450-dependent palmitate oxidation activity used as the starting material for further purification of the monoxygenase complex by gel filtration chromato-
tography. The partial purification of the monoxygenase complex by Sephadex G-200 chromatography has been described previously (Miura and Fulco, 1974). The procedure employing Sephacryl S-300 is described in previous results (Fig. 1). Both gel filtration method-yielded preparations that were free of epoxide hydrolase activity, had similar specific activities for palmitate hydroxylase, and contained about the same levels of cytochrome P-450 (40-70 pmol/mg of protein). Nevertheless, there were also differences between these two preparations and these are considered in context under “Results” and “Discussion.” For the experiments reported in this paper, pooled column fractions, containing essentially all of the monoxygenase activity, were used and designated G-200 or S-300 enzyme. These preparations had specific activities of about 20 nmol of palmitate hydroxylated/min/mg of protein compared to 6-7 for the starting (NH₄)₂SO₄ preparation. However, subsequent procedures (dialysis, lyophilization, aging in the frozen state, freeze-thawing) all resulted in small, variable losses in activity. When the specific activity of a preparation had been reduced by about half of the original it was discarded.

Assays for Monoxygenase Activities—Incubations were carried out in screw-caps tubes at 20 °C with gyrotary agitation. The standard assay mixture contained 0.10 M potassium phosphate buffer (pH 8.0), 200 μM potassium [1-\(^{14}\)C]palmitoleate (60,000 dpm) with either G-200 or S-300 enzyme protein. Incubations were initiated by the addition of substrate. After 5 min, the reaction was terminated by the addition of an equal volume of 58% NH₄OH. Tubes were then capped and heated in an oven at 80 °C for 8-12 h. Under these conditions, epoxypalmitate was converted to vicinal aminohydroxypalmitate while palmitoleate or hydroxypalmitoleate remained chemically un-
changed (Michaels et al., 1980). After the assay tubes were removed from the oven and cooled to 0 °C, the contents were acidified with 2 N HCl and extracted several times with ether to quantitatively remove palmitoleate and hydroxypalmitoleate. These components were sepa-
rate by silica gel column chromatography (Miura and Fulco, 1974) and the radioactivity of each was determined by scintillation counting. The cis-aminohydroxypalmitate, present as the hydrochloride, re-
mained in the aqueous phase during ether extraction. A portion of this aqueous phase was counted to determine the radioactivity or-
iginally present as epoxypalmitate. For the assessment of monoxygenase activity with nonradioactive substrates, spectrophotometric de-
termination was performed by a combination high pressure nitrogen bomb-sion technique as described previously (Matson et al., 1977) using a Beckman Model 35 UV-Vis spectrophotometer with reaction volumes of 2.25-
4.0 ml. Substrate-dependent NADPH oxidation was taken as the difference in the reaction mixture measured in the presence of fatty acid in a reaction mixture containing enzyme, NADPH, and buffer. The validity of this assay as a measure of monoxygenase activity was determined for each new enzyme preparation by using [1-\(^{14}\)C]palmitate and [1-\(^{14}\)C]palmitoleate as substrates and directly comparing substrate-dependent NADPH oxidation with radioactive product formation. With all preparations, initial rates of NADPH oxidation (first 2 min after NADPH addition), substrate-dependent NADPH oxidation, and radioactive product formation coincided. After that time, however, the rate of radioactive product formation decreased more rapidly than did the rate of substrate-dependent NADPH oxidation. A brief consideration of this phenomenon is included under “Discussion.” The specific monoxygenase activity for any preparation was always expressed as nanomoles of [1-\(^{14}\)C]palmitate hydroxylated/min/mg of protein using standard assa-y conditions.

Other Analytical Procedures—The cytochrome P-450 content of monoxygenase preparations was determined by the CO-difference spectrum as described previously (Matson et al., 1977) while the protein content was measured by the method of Warburg and Christian (1941). The distribution of radioactivity on thin layer silica gel plates was determined by scanning on a Model 7200 Packard radi-
ocromatogram scanner (Matson et al., 1977). Scintillation counting was performed on a Beckman Model LS 8100 liquid scintillation system.

RESULTS

Co-purification of Hydroxylase and Epoxidase Activities—Once we recognized that our partially purified fatty acid hydroxylase preparation from B. megaterium could also cata-
yze the epoxidation of palmitoleic acid (Buchanan and Fulco, 1978), we examined the basic co-factor requirements for epoxidation. As previously shown for the cytochrome P-
450-catalyzed hydroxylase reaction (Miura and Fulco, 1974), both molecular oxygen and NADPH were absolute require-
ments for epoxidation activity; NADH could not substitute in either reaction. Furthermore, when O₂ or NADH were made limiting, the rates of hydroxylation and epoxidation of pal-
mitoleate were affected to the same degree, an indication that both reactions might be catalyzed by the same enzyme or by very similar enzymes. This view was strengthened when we found that carbon monoxide inhibited epoxidation as well as hydroxylation. We thus began a systematic examination of the fractionation techniques we routinely use to prepare appro-
imately 100-fold purified cytochrome P-450 complex (see “Experimental Procedures”) to determine whether, at any point, there was some indication of separation of hydroxy-
lation and epoxidation activities. The ratio of the two activities remained constant in protein fractions during the centrifuga-
tion (NH₄)₂SO₄ steps. About 75% of the total activity for either hydroxylation or epoxidation of palmitoleic acid was concentrated in the protein precipitate obtained at 40-55% (NH₄)₂SO₄ saturation of the 27,000 × g supernatant. Fractiona-
tion of this precipitate on Sephadex G-200, a procedure that also removes the last traces of epoxide hydrolyase activity (Michaels et al. 1980) again gave no separation of the two monoxygenase activities. Purification of the 40-55% (NH₄)₂SO₄ precipitate on Sephacryl S-300 (which gives a protein-activity profile different than that obtained on Sepho-
dex G-200) was also examined with similar results. As Fig. 1 shows, the elution profiles of the two activities (hydroxylation and epoxidation), based on radioactive product formation from [1-\(^{14}\)C]palmitoleate, were superimposable. Hydroxylation of palmitate, assayed by taking the initial rate of substrate-
dependent NADPH oxidation (data not shown) also gave the same elution profile. It may also be noted (Fig. 1) that, despite apparent partial splitting of the monoxygenase activity peak, the hydroxylation to epoxidation ratio of 2:1, obtained with the starting 40-55% (NH₄)₂SO₄ precipitate, is retained throughout the Sephacryl fractionation.

Effect of Incubation Conditions on the Hydroxylation and Epoxidation of Palmitoleate—Although the correspondence between hydroxylation and epoxidation activities during partial purification support the concept that the two activities were catalyzed by the same enzyme, such evidence was certainly not compelling. The next test for correspondence between the two activities involved an examination of the effects of changes
in product ratios. Several other explanations of a more complex nature (i.e. involving different basic mechanisms of oxygenation) were considered much less likely on theoretical grounds.

**Inactivation and Inhibition of Monoxygenase Activities—**
The next experiment, involving heat inactivation of the monoxygenase activities of the P-450 complex preparation, was, again, designed to test for the presence of two separate enzymes. As Fig. 5 shows, the rates of inactivation for hydroxylation and epoxidation change dramatically between 40 and 45 °C with rates at the higher temperature being greater by 1 order of magnitude. Nevertheless, all rates are first order and a least squares fit of the data gives decay rates for hydroxylation and epoxidation activities that are almost exactly equal. These results, in addition to the results presented in Figs. 1–3, argue strongly against the possibility that different P-450 complexes catalyze the two reactions and indicate that other explanations must be sought to interpret the pH data. Thus, we next consider the idea that one enzyme containing two active sites could separately catalyze either hydroxylation of fatty acids at the terminal three methylene carbons or epoxidation of palmitoleate at the 9-10 double bond. Since carbon monoxide interacts directly with the active site of P-450 enzymes (Sato and Omura, 1978), we looked for differential inhibition of the two reactions at increasing concentrations of
Fatty Acid Epoxidation by a Cytochrome P-450 System

FIG. 4. Oxygenation of palmitoleate as a function of pH. Each incubation mixture, at 20 °C, contained 0.10 M potassium phosphate buffer of the pH indicated, 60 μM potassium [1-14C]palmitoleate, 200 μM NADPH, and 2.2 mg/ml of S-300 enzyme protein. Reactions were initiated by the addition of NADPH and terminated after 1 min. The results are expressed both as the sum of radioactive oxygenated products (O) and as the ratio of labeled hydroxypalmitate to epoxy-palmitate (O) at each data point on the pH curve.

CO. As indicated in Fig. 6, the inhibition curves for palmitoleate hydroxylation and epoxidation with increasing CO concentration are very close. Furthermore, the CO/O2 ratio required for half-maximal inhibition of palmitoleate oxygenation is the same as that observed previously for CO inhibition of palmitate hydroxylation (Hare and Fulco, 1976; Ho and Fulco, 1978). This experiment, then, supports the concept that only one P-450 enzyme with one active site catalyzes both reactions.

The next experiment was designed to test the possibility that two distinct substrate-binding sites, positioning the substrate differently with respect to the active site, were involved in hydroxylation and epoxidation. We reasoned that, if such were the case, palmitate, which can only be hydroxylated, would inhibit these two reactions to a different degree. In fact, as Fig. 7 illustrates, unlabeled palmitate, added to reaction mixtures containing the monooxygenase preparation and [1-14C]palmitoleate, inhibits both hydroxylation and epoxidation to the same degree. Furthermore, since 50% inhibition occurs at about a 1:1 ratio of palmitate to [1-14C]palmitoleate, the Km values for these two substrates are approximately the same. Finally, in another experiment (data not shown) we found that the ratio of hydroxy product to epoxy product remained constant as the [1-14C]palmitoleate concentration was varied between 5 and 160 μM and the reactions, in each case, were allowed to run to completion. The results of this and the preceding experiment (Fig. 7) are incompatible with the two-binding-site hypothesis. The remaining two stated alternatives, consistent with the pH data, include a pH-mediated change in the geometry of the active-site region or else the existence of several pH-dependent substrate-binding conformations near the middle of the acyl chain that could favor one product over another. Arguments on the relative merits of these two alternatives are considered in some detail under "Discussion."

Effect of Substrate Structure on Hydroxylation and Epoxidation—Once we were satisfied that a single P-450-compo-
nent (with one active site and one substrate-binding site) catalyzed both epoxidation and hydroxylation, we began an investigation of the effects of substrate structure on these two reactions. We first determined whether enzymatically hydroxylated palmitoleate could be epoxidized and, conversely, whether epoxypalmitate could be hydroxylated; in either case to form the hydroxyepoxy derivative. As Fig. 8 shows, an enzymatically prepared isomeric mixture of 1-14C-labeled 15-, 14- and 13-hydroxypalmitoleate is a much poorer substrate for epoxidation than is [1-14C]palmitoleate. On the other hand, both cis- and trans-9,10-epoxypalmitate are reasonably good substrates for hydroxylation. Either geometric isomer inhibited the hydroxylation of [1-14C]palmitate by 15-20% when added in an equal molar amount (data not shown).

Table I indicates the distribution of hydroxy and epoxy products formed from various monounsaturated fatty acids. Although the initial rates of substrate-dependent NADPH oxidation are remarkably similar for the tested fatty acids (data not shown), the final distribution of hydroxylation and epoxidation products varies widely with chain length and the position and geometry of the double bond. Thus, (Table I) palmitoleate gives 2.5 times more epoxy product than does its trans isomer while cis-10-hexadecenoate is more than twice as effective as a substrate for epoxidation than is its 18-carbon homolog, cis-12-octadecenoate. Similar comparisons can be made for other substrate pairs, an indication that, although the overall rate of oxygenation is relatively insensitive (at saturating substrate levels) to structural variation near the center of the fatty acid chain, the rate of monounsaturated fatty acid epoxidation is exquisitely responsive to such variations.

**DISCUSSION**

Multiple forms of cytochrome P-450 have been isolated from liver microsomes (Haugen et al., 1975), from adrenal cortex mitochondria (Suhara et al., 1978) and from the bacterium *Rhizobium japonicum* (Appleby and Daniel, 1973; Dus et al., 1976). Suggestive evidence has been obtained for similar heterogeneity from several other sources (Capdevila et al., 1975; Betz et al., 1976). The cytochrome P-450-dependent fatty acid hydroxylase obtained by us from *B. megaterium* 14581 and the cytochrome P-450_{13368}-dependent steroid hydroxylase obtained by Berg et al. (1975; 1976) from another strain, *B. megaterium* 13368, have also proved to be distinctly different by a number of criteria (Matson et al., 1977). Therefore, when we discovered that our fatty acid hydroxylase preparation also catalyzed the epoxidation of palmitoleic acid, we at once considered the possibility that two different P-450 monoxygenases might be involved. The results of the experiments reported in this paper, however, strongly support the concept that both hydroxylation and epoxidation are catalyzed by an identical cytochrome P-450 complex utilizing the same active and binding sites. Although no one experiment unequivocally demonstrates this point, the cumulative evidence is quite convincing. This evidence can be summarized as follows.
1. Hydroxylation and epoxidation activities show exact correspondence during the purification procedures utilized to date (Fig. 1).

2. The two activities varied in the same way in response to changes in the order of reactant addition (Fig. 2) or protein concentration (Fig. 3).

3. The activities for hydroxylation and epoxidation decrease at exactly the same rates in response to temperature inactivation of monooxygenase preparations at 40 and 45°C (Fig. 5).

4. Both activities exhibit the same sensitivity to inhibition by CO (Fig. 6).

5. Finally, the hydroxylation and epoxidation of palmitoleate are inhibited to the same degree by palmitate (Fig. 7), a substrate that can be hydroxylated but not epoxidized.

6. The only experiment that does not clearly support the concept that hydroxylation and epoxidation of palmitoleate by the B. megaterium monooxygenase preparation are catalyzed by the same enzyme complex is the one showing that these two activities do not exhibit the same dependence on pH. Nevertheless, this finding is not incompatible with the one-enzyme hypothesis and, in the light of the other experiments reported here, must logically be interpreted from this viewpoint. As noted under "Results," an analysis of all of the experimental data seems to leave only two likely explanations for the results of the pH experiment: either there is a pH-mediated change in the geometry of the active site region or else there are two or more pH-dependent substrate-binding conformations. In either case, as pH decreases, there must be a changing spatial relationship between the active site and the double bond of the substrate that increases the probability of epoxidation relative to hydroxylation. We think, however, that the results of previously published experiments on the positional specificity of palmitate hydroxylation by a partially purified monooxygenase preparation from B. megaterium 14681 (He and Fulco, 1976) argues strongly against a change in active-site geometry. Thus, it was shown that, over a pH range of 4.95–10.0, there was no significant change in the distribution of the three hydroxypalmitate isomers formed as products, although there was a 5-fold change in monooxygenase activity. This provided strong support for our previously stated hypothesis (Miura and Fulco, 1975) that, in addition to a polar binding site that interacts with the carboxyl terminus, there is a hydrophobic pocket adjacent to the active site that binds and sequesters the terminal methyl group of the fatty acid substrate and rigidly positions the next three methylene carbons in a set geometry at the active site. On the other hand, we have recently published evidence (Matson et al., 1980) indicating that there is little hydrophobic interaction between the enzyme and the midchain region of the substrate chain and that the steric requirements in this region are not rigorous. This finding is quite compatible with the concept that the double-bond (midchain) region of palmitate may interact or bind loosely with the enzyme in several conformations that would increase or decrease the probability that it would be in position for epoxidation during the oxygenation reaction. It would follow that the conformation(s) involving a higher probability of epoxidation must be favored by a lower pH (Fig. 4). In this regard, it should be noted that hydroxylation of saturated fatty acids is limited to the ω -1, ω -2, and ω -3 positions (Miura and Fulco, 1975), although, conceivably, they could also assume conformations that would position methylene carbons of the midchain region near the active site. The fact that palmitic acid is not hydroxylated at the 9 or 10 position while palmitoleic and palmelaidic acids undergo epoxidation at these carbons (Table I) may reflect the much enhanced reactivity of the carbon-carbon double bond. White et al. (1979) found that methycyclohexane was hydroxylated in all positions by the purified liver microsomal cytochrome P-450 system but that oxygenation of cyclohexene was restricted to epoxidation of the double bond and hydroxylation of the allylic positions in about equal proportions.

7. We found that all unsaturated fatty acids, including those containing a double bond or a hydroxy group, have about the same V_max. This implies that, for saturating levels of these substrates, the rate-limiting step in oxygenation does not involve substrate structure, product release, or the ratio of hydroxylation to epoxidation. Thus, the position and geometry of the double bond in a monounsaturated substrate may dramatically affect the relative rate of epoxidation but the combined rate of hydroxylation and epoxidation is essentially a constant independent of this parameter. Although more unsaturated substrates must be examined before predictive relationships can be established between location and geometry of a double bond and its rate of epoxidation, most of the relationships seen in Table I can be rationalized on the basis of the enzyme-substrate binding model discussed above. We assumed, based on the known positional specificity of hydroxylation (Miura and Fulco, 1975) that the ω -2 carbon of the bound substrate was closest to the active site of the P-450 enzyme. We then constructed molecular models (CPK Models, Ealing Corp., Cambridge, MA) of the substrates shown in Table I and examined various conformations that would bring the double bond hydrogens close to the hydrogens of the ω -2 carbon. With palmitoleate (34.5% epoxidation) the hydrogens of both double bond carbons could be positioned at the active site. With cis-10-hexadecenoate (24.5% epoxidation) only the hydrogen of the double bond carbon in the 11-position could be arranged adjacent to the hydrogen of the ω -2 carbon. The double bond hydrogens of oleate (2.6% epoxidation), 12-hydroxy-cis-9-octadecenoate (1.1%) and cis-5-tetradecenoate (0.7%) were prevented by steric factors from intimate association with the active site. In addition, the 14-carbon substrate, anchored to the enzyme surface at the methyl-terminal region and the carboxyl group, may not have sufficient chain length to permit significant flexibility in the midchain region. The double bond of cis-12-octadecenoate (11.4% epoxidation) can assume the same proximity to the active site as that of its 16-carbon homolog (cis-10-hexadecenoate) and, as one would expect, cis-Δ 12-C16 is a much better substrate for epoxidation than oleate. Nevertheless, it is less active by a factor of 2 than its C16 homolog, a consequence, perhaps, of more competing binding conformations in the midchain region as allowed by the additional two carbons. The relative epoxidation activities of the two trans-monounsaturated fatty acids tested (Table I) could not be easily rationalized using our conformation models. Trans-9-hexadecenoate was 40% as active as its cis isomer as an epoxidation substrate, but trans-9-octadecenoate had twice the epoxidation activity of oleate. In the course of our study of fatty acid epoxidation and its relationship to the cytochrome P-450-catalyzed hydroxylation of fatty acids, there were several more general observations that perhaps merit comment. One point of interest involves the observation that, although the rate of substrate-dependent NADPH oxidation and substrate oxygenation coincides for the first several minutes after reaction has been initiated in our system with NADPH, eventually the rate of NADPH oxidation may exceed by 50% the concurrent rate of substrate oxygenation. Similar observations, involving the uncoupling of NAD(P)H oxidation from substrate hydroxylation have been reported by Ribbons and Ohta (1970) for cocin hydroxylase from Pseudomonas putida, by White-Stevens and Kam (1970) for bacterial salicylate hydroxylase, and by Nord-
bloom and Coon (1977) and Vermilion and Coon (1978) for the purified liver microsomal P-450 system. In each case the reduction of \( \text{O}_2 \) by \( \text{NAD(P)}\text{H} \) to form \( \text{H}_2\text{O}_2 \) was implicated as the uncoupling reaction. It seems quite possible that this reaction may also explain the partial uncoupling of NADPH oxidation from substrate oxygenation in our system, although we have not yet tested for the production of \( \text{H}_2\text{O}_2 \) during the oxygenation reaction.

Another observation that was quite reproducible was the partial resolution of oxygenase activity into two peaks when the 40–55% \( (\text{NH}_4)_2\text{SO}_4 \) fraction was further purified on Sephadex S-300 (Fig. 1). No peak splitting occurred when either the \( (\text{NH}_4)_2\text{SO}_4 \) precipitate or the S-300 preparation was chromatographed on Sephadex G-200 and no significant differences were observed in the hydroxylations:epoxidation or cytochrome P-450:oxygenase activity ratios when the two peak fractions were compared. The only difference we could detect was the appearance of a substrate-independent NADPH oxidation activity peak (Fig. 1, fractions 60–75) that coincided with the second S-300 oxygenase activity peak. On Sephadex G-200, all of the substrate-independent NADPH activity was found in one peak corresponding to fractions 30–55 on S-300. Since the second S-300 oxygenase peak presumably represents a lower molecular weight form of the complex, it seems unlikely that it was formed by the addition of another component (such as a second reductase unit) to the native complex. We are presently attempting to purify further the S-300 complex preparation by other chromatographic methods and intend to investigate this phenomenon in the process.

Acknowledgments—We wish to acknowledge the excellent assistance of Johnny Sight and Sue Bony in many of the experiments reported here. We also thank Linda Narhi for her active participation in certain aspects of this research and Ellen James for the preparation of the figures for publication.

REFERENCES


