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Enzymic ω-Oxidation: Stoichiometry of the ω-Oxidation of Fatty Acids[1]

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The stoichiometry of the ω-oxidation of fatty acids catalyzed by the inducible enzyme system of *Pseudomonas oleovorans* was determined. Assay methods are described for the polarographic measurement of oxygen uptake and the spectrophotometric measurement of DPNH oxidation. The development of these assay methods for the ω-hydroxylase has permitted the determination of the stoichiometry of the ω-hydroxylation of fatty acids. The reaction is a mixed function oxidation and the following equation is sufficient to describe the stoichiometry of the reaction catalyzed by the enzyme system:

$$\text{DPNH} + \text{H}^+ + \text{laurate} + \text{O}_2 \rightarrow \text{DPN}^+ + \text{H}_2\text{O} + \omega\text{-hydroxylaurate}$$

The classification as mixed function oxidases of enzymes which introduce a hydroxyl group into substrates has rested mainly on the demonstration of a requirement for molecular oxygen and for a source of reducing equivalents (1). A basic assumption in the preliminary work with hydroxylating enzymes is that the stoichiometry of the reaction will be shown to conform to the relationship described by Mason (1). In a few cases, such as steroids, the incorporation of one of the atoms of the O^{18} oxygen molecule into the hydroxyl group of the product of the hydroxylation reaction has been demonstrated (2–4). The stoichiometry of the reaction has been established for only a few of the mixed function oxidases. The stoichiometric relationship of TPNH, O_2, and phenylalanine utilized and tyrosine formed during the mixed function oxidation reaction catalyzed by phenylalanine hydroxylase, was first shown by Kaufman (5). The relationship between oxygen utilized, TPNH oxidized, and cortexolone formed was measured for the reaction catalyzed by the C21-hydroxylase of adrenal cortex (6). The stoichiometric relationship between two or more of the substrates has been established in the mixed function oxidation of camphor by *Pseudomonas putida* (7), and for the *N*-demethylation of aminopyrine by liver microsomes (8). However, in each of these cases the measurement of one or more of the other components of the reaction was omitted.

The ω-oxidation of fatty acids and hydrocarbons by the inducible enzyme system

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of *Pseudomonas oleovorans* has been shown to require both DPNH and molecular oxygen (9–11). The hydroxylase system has been separated into three enzyme components (12, 13). One of the enzymes involved is rubredoxin, a nonheme iron protein without labile sulfur (14) and another is a DPNH-specific rubredoxin reductase which can be completely replaced by spinach ferredoxin-TPN reductase (12). The third protein fraction required is the ω-hydroxylase: the nature of its prosthetic group has not been identified. A similar alkane hydroxylase system requiring three enzyme components has been shown to be present in *Pseudomonas desmolytica* (15). The DPNH-dependent rubredoxin reductase of this organism was shown to require FAD for maximal activity (15). The data presented in this paper establishes the stoichiometry of the components of the ω-oxidation reaction of fatty acids catalyzed by the enzyme system for *P. oleovorans*, and shows that the reaction can be classified as a mixed function oxidation. The following equation will be shown to be sufficient to describe the stoichiometry of the reaction:

$$\text{DPNH} + \text{H}^+ + \text{O}_2 + \text{R—CH}_3 \rightarrow \text{DPN}^+ + \text{H}_2\text{O} + \text{R—CH}_2\text{OH}$$

MATERIALS AND METHODS

Growth of the bacterium. P. oleovorans was grown on a rotatory shaker on a mineral salts medium which contained, in grams per liter, the following substances: $MgSO_4$, 0.2; $CaCl_2$, 0.02; $FeCl_3$, 0.02; $(NH_4)_2SO_4$, 1.0; KH_2PO_4, 0.5; Na_2HPO_4, 3.5; yeast extract, 0.02; and, in addition, 30 g of hexane essentially as described previously (16). The growth of the organism was followed turbidimetrically at 550 mμ and the cells were harvested in the late log phase of growth. The cells were washed twice with 1/20th of the original volume of the growth medium, using 0.1 M potassium phosphate buffer, pH 7.4. The same buffer was used throughout this study unless otherwise stated. The cells were "starved" by vigorous aeration in the buffer on a rotatory shaker for 1 hr. The starved cells were harvested and washed twice with buffer and stored at 3° until used. The protein concentration was determined by the biuret method.

Preparation of enzyme fractions. The protein concentration of the whole cell suspension was adjusted to 30 mg/ml with the buffer and the cells were broken by sonication with a Branson sonifier at full output for 3 min. The cell debris and unbroken cells were removed by centrifugation at 8000*g* for 10 min and then at 30,000*g* for 15 min. The DPNH oxidase fraction was removed by centrifugation at 100,000*g* for 30 min leaving the ω-hydroxylase system in the supernatant solution. The ω-hydroxylase system could be further fractionated by centrifugation at 200,000*g* for 120 min. Based on the results of previous studies, the ω-hydroxylase was assumed to be in the pellet while the rubredoxin and reductase were predominantly in the supernatant solution.

Oxygen uptake was measured polarographically with a Clark oxygen electrode essentially as described by Estabrook (17). The change in H^+ concentration was measured simultaneously with oxygen uptake using a recording pH meter essentially as previously described (18). DPNH oxidation was followed at 340 mμ with an Aminco-Chance dual wavelength, wavelength-scanning spectrophotometer. DPNH was added to both the reference and sample cuvette so that the initial optical density difference at 340 mμ between the reference and sample was zero.

The lauric acid used was obtained from Applied Science Laboratories and was 99.8% pure by gas liquid chromatography. The *n*-hexane used for growth of the organism was Fisher spectral quality. Highly purified spinach TPNH: ferredoxin reductase, prepared by a modification of the method of Shin, Tagawa, and Arnon (19) was a gift from Mr. Gordon P. Foust.

RESULTS

Both fractions prepared by differential centrifugation were required for oxygen uptake in the presence of DPNH and either *n*-hexane, *n*-octane (20), or sodium laurate. Figure 1 is a typical example of an experiment with laurate as substrate and demonstrates the dependency of oxygen uptake on the presence of both protein fractions. These fractions, which were prepared by differential centrifugation, are probably comparable to fractions A and B previously prepared from this organism (9). The small rate of oxygen uptake in the presence of the 200,000*g* pellet, DPNH, and laurate, probably represents a slight contamination of the pellet with the soluble reductase and rubredoxin.

The effect of the depletion of the oxygen dissolved in the reaction mixture on the rate of the reaction can be used to estimate the K_m value of the hydroxylase for oxygen. The concentration of oxygen remaining when

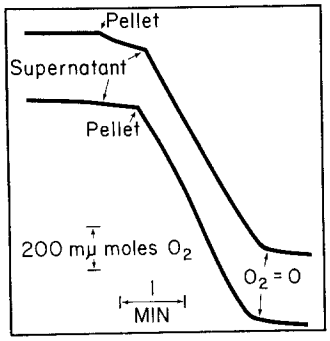

FIG. 1. Requirement of two fractions for oxygen uptake: The reaction was performed in the O_2 electrode vessel with the buffer, 1 μmole of sodium laurate and 1.2 μmoles DPNH with a final volume of 3.2 ml. At the appropriate times, the supernatant fraction (4.0 mg of protein) and the pellet (4.9 mg of protein) were added. The observed rate of O_2 uptake was 0.66 μmole per minute in the presence of both the supernatant and pellet fractions.

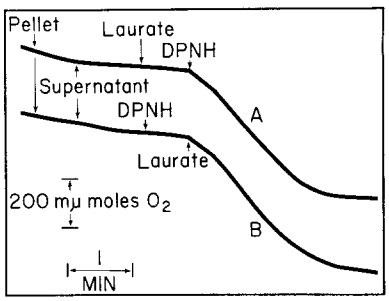

FIG. 2. Oxygen uptake as a measure of limiting DPNH or laurate concentration: The conditions were essentially as described in Fig. 1, except that for curve A, 650 mμmoles of DPNH were added and for curve B 600 mμmoles of sodium laurate were added. In each of the experiments the other components were present in excess.

the rate slows to one half of the maximal rate is equal to the K_m. The concentration of oxygen giving one half of the maximal velocity is approximately 10 μM. At a concentration this low, the rate of diffusion of O_2 across the membrane can become limiting and so the K_m of the hydroxylase for oxygen can be estimated to be less than 10 μM.

Figure 2 shows the stimulation of oxygen uptake upon addition of DPNH to the complete hydroxylation system with the 200,000*g* supernatant solution and laurate in excess. Figure 2 also shows the stimulation of oxygen uptake upon addition of laurate to the complete system with DPNH and the 200,000*g* supernatant solution in excess. In curve A, the molar ratio of DPNH added to O_2 taken up was 1.0 and in curve B, the molar ratio of sodium laurate added to O_2 taken up was 1.0. The rate of oxygen uptake with the complete system present was not affected by the addition of catalase to the reaction mixture, indicating that the oxygen uptake was a measure of hydroxylation and not due to the generation of H_2O_2. The small rate of oxygen uptake in the absence of added laurate probably represents the slow hydroxylation of endogenous substrate. Many experiments, not described in this paper, have shown that the rate of oxygen uptake in the absence of added substrate is a function of the care taken in washing and starving the cells prior to use. The better washed and starved the cells, the lower the endogenous rate of oxygen uptake. Even in the absence of added substrate for hydroxylation, the DPNH-dependent oxygen uptake corresponds in molar amount to the oxygen utilized. An analysis like that described for the determination of the K_m for oxygen can be used to determine the K_m of the system for laurate and DPNH. With a limiting concentration of DPNH added and the other components in excess, the effect of the depletion of DPNH on the rate of oxygen uptake is observed. The K_m of the system for DPNH can be approximated from experiments such as this to be less than 15 μM. The experiment of curve B shows that the K_m for laurate is approximately 30 μM. The K_m for laurate has been determined by measuring the effect of added laurate on the rate of hydroxylaurate formation. The value obtained previously was 33 μM (11) which is excellent agreement with the value obtained above.

The linearity of the oxygen electrode assay for the hydroxylase is shown in Fig. 3. It can be seen that the rate is linear with increasing amounts of hydroxylase added over a fivefold range. The specific activity of the hydroxylase fraction calculated from a plot such as this is 112 mμ moles per minute per milligram of protein.

The rate of oxygen utilization was suffi-

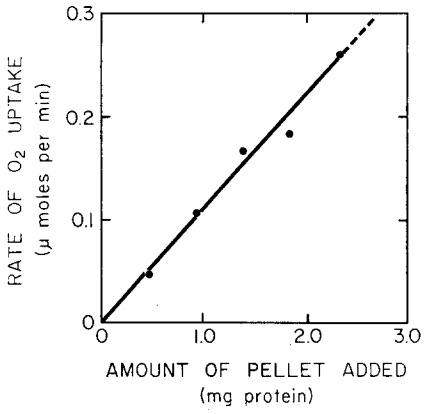

FIG. 3. Linearity of the polarographic assay for the hydroxylase. The experiment was performed in the O_2 electrode vessel essentially as described in Fig. 1, with varying concentrations of the hydroxylase (pellet) fraction. The rate plotted is the difference in the observed rate of O_2 uptake in the presence and absence of the hydroxylase. The other components were in excess.

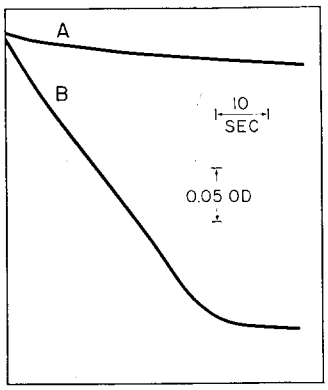

FIG. 4. Spectrophotometric assay of the ω-hydroxylase: The reaction mixture contained buffer, supernatant enzyme fraction (4.6 mg of protein), and the hydroxylase fraction (1.5 mg of protein). The final volume was 3.0 ml. The reaction was initiated by the addition of 150 mμmoles of DPNH. Curve A represents the rate of DPNH oxidation in the absence of added sodium laurate. Curve B represents the rate of DPNH oxidation in the presence of 1 mM sodium laurate.

ciently rapid so that the rate of DPNH oxidation could be easily measured spectrophotometrically. Figure 4 shows an experiment in which the rate of DPNH oxidation was measured spectrophotometrically at 340 mμ in the presence (B) and absence (A) of laurate. The rate of DPNH oxidation under these conditions was shown by other experiments to be dependent upon the amount of hydroxylase fraction added (Fig. 5). The rate of the reaction was, therefore, a true measure of the maximal velocity of the hydroxylation reaction. The rate of oxygen utilization per milligram hydroxylase protein, as measured with an oxygen electrode, was found to be equal to the rate of DPNH oxidation per milligram hydroxylase protein measured spectrophotometrically. These results clearly establish that the ratio of the rates of DPNH and oxygen utilization under these conditions is one, and that the reaction conforms to the definition of a mixed function oxidation reaction. Figure 5 shows a plot of the effect of increasing the amount of hydroxylase fraction on the rate of DPNH oxidation, as measured spectrophotometrically. The assay method is linear with increasing amounts of hydroxylase over a fivefold range of enzyme concentration.

The oxidation of 1 mole of DPNH should be accompanied by the utilization of a hydrogen ion equivalent, and the solution, therefore, should become more alkaline. This parameter was followed using an oxygen electrode vessel which had been modified so

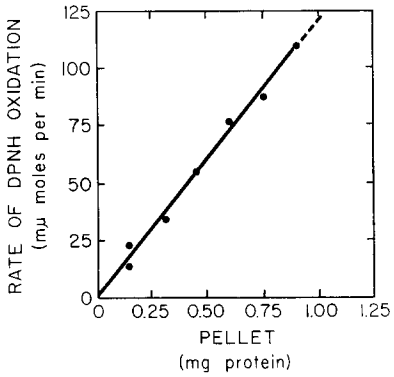

FIG. 5. Linearity of the spectrophotometric assay for the hydroxylase fraction: The reaction mixture contained 0.1 M phosphate buffer pH 7.4 and 1 mM sodium laurate in both the sample and reference cuvettes. The final volume of the reaction mixture was 3.0 ml. The sample cuvette contained the supernatant enzymes (4.6 mg of protein) and the indicated amount of the hydroxylase (pellet) fraction. The reaction was initiated by the addition of 150 mμmoles of DPNH. DPNH oxidation was followed at 340 mμ using an Aminco-Chance split-beam spectrophotometer.

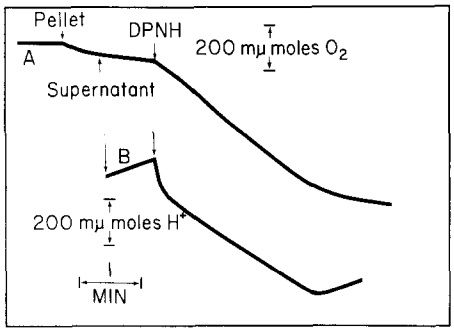

FIG. 6. Stoichiometry of O_2 and H^+ uptake stimulated by DPNH. The experiment was performed essentially as described in curve A of Fig. 2 except that the O_2 electrode vessel was fitted with a small combination electrode for measuring pH changes in the medium. The reaction mixture contained 20 mM Tris buffer, pH 7.4, hydroxylase fraction (2.6 mg of protein), and the supernatant fraction (4.0 mg of protein) in a final volume of 5.5 ml. The reaction was initiated by the addition of 0.7 μmole of DPNH. At the end of each experiment, the H^+ uptake was standardized by the addition of 0.5 μmole of a standard solution of oxalic acid.

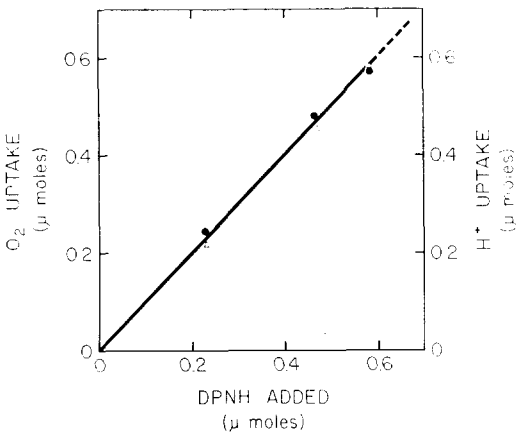

FIG. 7. Linearity of the amount of O_2 and H^+ uptake with respect to DPNH added: The results from experiments such as those described in Fig. 6 are plotted with △ representing H^+ versus DPNH added and ● representing O_2 uptake versus DPNH added.

that a combination electrode for measuring the pH of the solution could be inserted simultaneously with the oxygen electrode. As can be seen in Fig. 6, there was hydrogen ion uptake (downward deflection) which paralleled and equalled the amount of oxygen uptake and the amount of DPNH added. The drift of the baseline toward a lower pH can be attributed to the hydrogen ion release, due to the action of nucleases. The fractions used for these studies were crude and contained large amounts of nucleic acid, as evidenced by the ratio of optical density at 280 mμ to that at 260 mμ. The baseline drift was not so large as to be prohibitive for this type of study, and the rate of change of the hydrogen ion concentration returned to the original value after oxygen uptake stopped. The amount of hydrogen ion uptake dependent on hydroxylation could be calculated by determining the difference in pH between the initial and final states and assuming the baseline drift continued at a constant rate during hydroxylation. Figure 7, which is a plot of oxygen and hydrogen ion uptake versus the amount of DPNH added, describes the results of several such experiments. The ratio of O_2:DPNH:H^+ is one, indicated by the fact that the two lines are superimposable. Figure 8 is a plot of oxygen uptake versus laurate added. The results show that the ratio of oxygen utilized to the amount of laurate added is very nearly one, again indicating that the enzyme is a mixed-function oxidase. This also indicates that the product is not further metabolized under these conditions.

In the results already presented it was necessary to assume that laurate had been converted to the hydroxylated product without further metabolism of the hydroxylau-

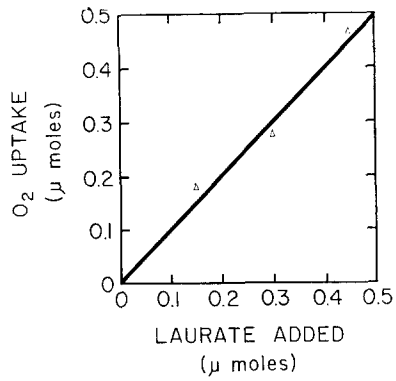

FIG. 8. Linearity of the amount of O_2 uptake with respect to laurate added. The amount of O_2 uptake dependent on sodium laurate added in experiments similar to those described in Curve B of Fig. 2 is plotted versus the amount of sodium laurate added.

TABLE I

STOICHIOMETRY OF LAURATE ω-HYDROXYLATION[a]

The three components were estimated in parallel experiments at 30° using 27 μg of homogeneous rubredoxin, highly purified spinach TPN-ferredoxin reductase (10 μg of protein), and partially purified ω-hydroxylase (0.2 mg of protein), 100 μmoles of Tris buffer, pH 7.0, 0.16 μmole of TPNH, and 0.2 μmole of laurate (saturating concentration) per milliliter, except that 0.4 μmole of laurate-1-^{14}C was used in the experiment measuring the hydroxylated product formed. TPNH disappearance was determined spectrophotometrically in a cuvette containing 1.0 ml of the reaction mixture, and oxygen uptake was measured by an oxygen electrode in a 3.0-ml reaction volume. The experiment with ^{14}C-laurate was carried out in a 1.0-ml reaction volume and the resulting radioactive ω-hydroxylaurate was isolated by silicic acid chromatography (10). The ratio is expressed as the amount of TPNH or O_2 utilized to ω-hydroxylaurate formed.

Component measured	Increase or decrease in reaction components (mμmoles per min per mg protein in hydroxylase fraction)	Ratio
TPNH	−50[a]	1.1
O_2	−52[a]	1.1
ω-Hydroxylaurate	+45[b]	—

[a] Laurate was omitted in control experiments.

[b] The ω-hydroxylase was omitted in a control experiment.

rate. Therefore a series of experiments was carried out in which the amount of ω-hydroxylaurate formed was determined using ^{14}C-labeled substrate, along with measurements of oxygen uptake and reduced pyridine nucleotide oxidation. The data presented in Table I were obtained using homogeneous rubredoxin and partially purified ω-hydroxylase, (13) as well as TPNH and highly purified spinach TPN-ferredoxin reductase in place of the usual crude bacterial DPNH-dependent reductase. The results clearly indicated that the amount of hydroxylaurate formed is equimolar with respect to the amounts of O_2 and TPNH consumed. In other experiments not described here it was shown that the presence of all three enzyme fractions as well as the appropriate substrates was necessary for enzymatic activity as judged by the radioactive, spectrophotometric, and polarographic assays.

DISCUSSION

The results presented in this paper on the stoichiometry of the ω-oxidation of fatty acids along with the results presented previously on the ω-oxidation of hydrocarbons (20) prove conclusively that the ω-hydroxylase *P. oleovorans* is a mixed function oxidase. The assay methods described in this paper for the determination of the stoichiometry of the reaction should prove to be of value in studying the ω-hydroxylase. The measurement of oxygen uptake during hydroxylation has been the most rapid and reproducible of these methods. These assay methods suffer from the problem of not specifically measuring the product of the hydroxylation reaction, but this problem can be partially overcome if a constant check is made on the molar ratio of DPNH oxidized to oxygen consumed. If this ratio varies from unity, the assay system can be assumed to be measuring something other than ω-oxidation.

The ω-hydroxylase of *P. oleovorans* remains essentially particulate, and even though much is known about the soluble enzymes which transfer electrons from DPNH to the hydroxylase, the nature of the functional group of the latter is unknown (13). The mixed function oxidase system of adrenal cortex, which is involved in steroid 11β-hydroxylation, has been shown to contain cytochrome P-450 and to require a flavoprotein and a nonheme iron protein (adrenodoxin) for activity (21). The methylene hydroxylation system of *P. putida* has been shown to require three enzymes; Katagiri, Ganguli, and Gunsalus (22) have recently identified a soluble cytochrome P-450 which is present in the hydroxylase fraction. The other two enzymes which are required are a flavoprotein and a nonheme iron protein (putidaredoxin) (23). The mixed-function oxidase system of *P. oleovorans* has also been shown to require three protein fractions, one of which is a nonheme iron protein (rubredoxin) which does not contain labile sulfur. The second fraction which contains the DPNH-rubredoxin reductase can be completely replaced by highly puri-

fied spinach ferredoxin: TPN reductase and the third fraction contains the ω-hydroxylase (12, 13).

In contrast to the *P. putida* system the partially purified ω-hydroxylase contains no detectable cytochrome P-450 (24) and its absence is further indicated by the failure of carbon monoxide to inhibit ω-hydroxylation (12). *P. oleovorans* grown on hexane and containing a competent ω-hydroxylation system has recently been shown to contain only one CO-binding pigment, cytochrome *o*. Cytochrome P-450 is therefore either totally absent or in so low a concentration as to not be detectable either in the whole organism or in the hydroxylase fraction prepared from it (25). Recently the ω-oxidation of *n*-octane by fractions prepared from *Corynebacterium* sp. was shown to be inhibited by CO. One of the fractions contained cytochrome P-450 (26). This finding would seem to indicate that there are at least two types of ω-hydroxylases functional in bacterial systems. On the other hand, cytochrome P-450 has been shown to function as an ω-hydroxylase in a soluble enzyme system from rabbit liver microsomes (27). The microsomal system also requires a TPNH-cytochrome P-450 reductase and a heat-stable factor for hydroxylation activity; a nonheme iron protein appears not to be required in contrast to the bacterial systems already described.

One parameter which remains to be measured for any mixed function oxidase is the amount of free energy released during hydroxylation. Recently, this void has been partiall by filledy measuring the energy released during camphor-stimulated oxygen uptake by a tightly coupled mixed function oxidase system prepared from *P. putida* which had been grown on camphor (28). The mixed function oxidation of camphor in Tris Cl^- at pH 7.4 was shown to release 97 Kcal per mole of oxygen consumed. The theoretical amount of free energy release was calculated as the sum of the free energy of reduction of one half mole of oxygen to water by DPNH (60 Kcal/mole) (29) and the free energy difference between a hydrophobic molecule and its hydroxylated product (47 Kcal/mole). The calculations were based on the difference in free energy of *n*-octane and *n*-octanol and cyclohexane and cyclohexanol (30). The theoretical and experimental values argee within experimental error for this type of determination.

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