CARBON MONOXIDE AND HYDROXYMERCURIBENZOATE SENSITIVITY OF A FATTY ACID (ω-2) HYDROXYLASE FROM BACILLUS MEGATERIUM

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SUMMARY: A soluble monooxygenase system from Bacillus megaterium, which hydroxylates fatty acids primarily in the (ω-2) position in the presence of NADPH and O_2, is 50% inhibited by carbon monoxide at a $CO:O_2$ ratio of 0.25 and 85% inhibited by 5 μM p-hydroxymercuribenzoate. The latter inhibition can be reversed by ferredoxin, cysteine or glutathione. These data are interpreted as indicating the involvement of a P-450-type cytochrome and a ferredoxin-type component in the hydroxylation. This system is not inhibited by superoxide dismutase, catalase or by moderate concentrations of various hydroxyl radical scavengers.

INTRODUCTION

Miura and Fulco (1,2) recently described a soluble monooxygenase system from Bacillus megaterium that converts long chain ($C_{15} \pm 3$) free fatty acids, alcohols and amides, primarily to their (ω-2) monohydroxy derivatives. They further showed (1) that passage of their crude hydroxylase preparation through Sephadex G-25 resulted in an 80% loss of specific activity, presumably due to the removal of a small protein component. The addition of Clostridium pasteurianum ferredoxin to this gel-filtered preparation completely restored hydroxylation activity. The activation of this preparation by an iron-sulfur protein suggests a similarity to several other bacterial and adrenal hydroxylases (3-5) and, parenthetically, distinguishes it from the hepatic system (6) which lacks the iron-sulfur protein.

Miura and Fulco (1) also showed that, when a combination of $(NH_4)_2SO_4$ fractionation and Sephadex G-200 chromatography of the crude hydroxylase preparation was substituted for Sephadex G-25 chromatography, the endogenous ferredoxin-replaceable component was apparently retained in the resulting 11-fold purified preparation. Unlike the G-25 preparation, there was no loss of

total activity during purification and the activity of the final preparation (G-200 enzyme) was not increased by added ferredoxin. The present report establishes a carbon monoxide sensitivity of the G-200 enzyme similar to other monooxygenases of the P-450 type and also demonstrates a reversible inhibition of the enzyme by p-hydroxymercuribenzoate.

MATERIALS AND METHODS

Enzyme preparation: The hydroxylase was prepared as described by Miura and Fulco (1) except that centrifugation was at 40,000 x g rather than at 34,000 x g and the supernatant was lyophilized after purification by $(NH_4)_2SO_4$ precipitation and before Sephadex G-200 chromatography. Such G-200 enzyme preparations had 15-25 times the hydroxylation activity of the starting 40,000 x g supernatant.

Assay for palmitate hydroxylation: All incubations were carried out in 15 ml test-tubes at 20° with gyratory agitation (250 rpm) for the duration of the incubation (usually 30 min). In addition to G-200 enzyme, each tube contained 100 μmoles of potassium phosphate buffer (pH 7.1), 0.5 μmoles of NADPH, 24 nmoles of sodium [1-^{14}C]palmitate and sufficient water or buffer to bring the final volume to 1.0 ml. For each experiment the specific activity of the G-200 enzyme is expressed in terms of the nmoles of palmitate hydroxylated per min per mg of enzyme protein under the standard assay conditions described above.

Materials: NADPH was purchased from P.L. Biochemicals, Inc., Clostridium pasteurianum ferredoxin from Worthington Biochemical Corp., [1-^{14}C]palmitate from New England Nuclear Corp., SOD* from Truett Laboratories, and catalase (thymol-free powder) from Sigma. The SOD had a specific activity of 3000$^+$ units per mg as defined by McCord and Fridovich (7) while catalase had a specific activity of 17,500 Sigma units.

RESULTS

Carbon monoxide inhibition of hydroxylation: Carbon monoxide, a characteristic

*Abbreviations: SOD = superoxide dismutase. PHMB = p-hydroxymercuribenzoate.

inhibitor of monooxygenase systems that involve cytochrome P-450 as an active component (8) was also a potent inhibitor of our (ω-2) hydroxylating system. As Fig. 1 shows, a $CO:O_2$ ratio of 0.25 resulted in a 50% inhibition of palmitate hydroxylation. At extremely low $CO:O_2$ ratios (0.05 - 0.075) however, there appeared to be a slight but quite reproducible stimulation of hydroxylation activity.

In other experiments, neither sodium azide nor o-phenanthroline (both at 5 mM) showed any effect on hydroxylation while 1 mM sodium cyanide caused a 10% inhibition and 3 mM a 50% inhibition of activity.

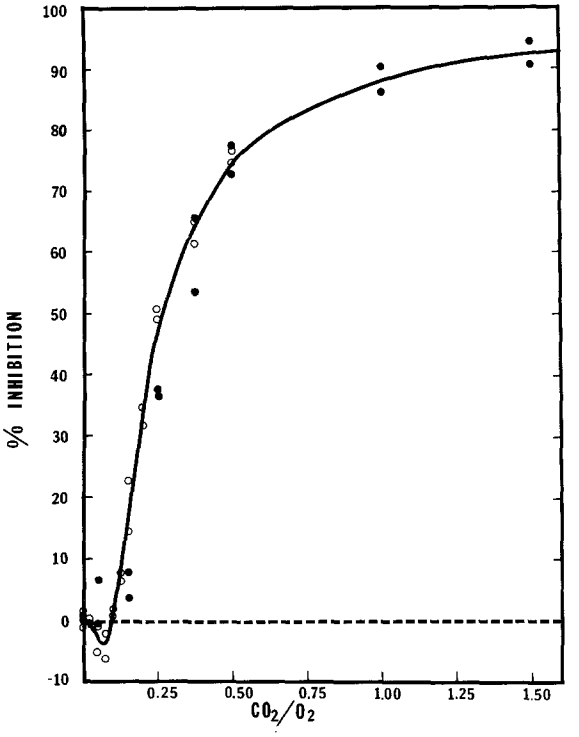

Figure 1. Inhibition of hydroxylation by carbon monoxide. Incubations in duplicate were carried out under standard assay conditions with a a G-200 enzyme preparation (320 μg protein/tube) having a specific activity of 1.58. Each tube was capped with a rubber serum stopper and contained 9 ml of air and 5 ml of a $CO-N_2$ mixture. The first experiment (•) defined the general shape of the inhibition curve while the second experiment (o) more clearly delineated the effects of low CO concentrations.

Reversible inhibition of hydroxylation by p-hydroxymercuribenzoate: The sulfhydryl-modification reagent, PHMB proved to be a very effective inhibitor of palmitate hydroxylation in the B. megaterium system. In typical experiments using 150-250 μg of enzyme protein per ml, 50% inhibition was achieved at PHMB concentrations of 3-4 μM and greater than 95% inhibition at 8-10 μM. At the lower PHMB concentrations, complete restoration of activity could generally be obtained by adding molar equivalent amounts of ferredoxin, cysteine or glutathione. However, at higher levels of PHMB, only cysteine and glutathione could completely restore activity. As Fig. 2 shows, the effectiveness of C.

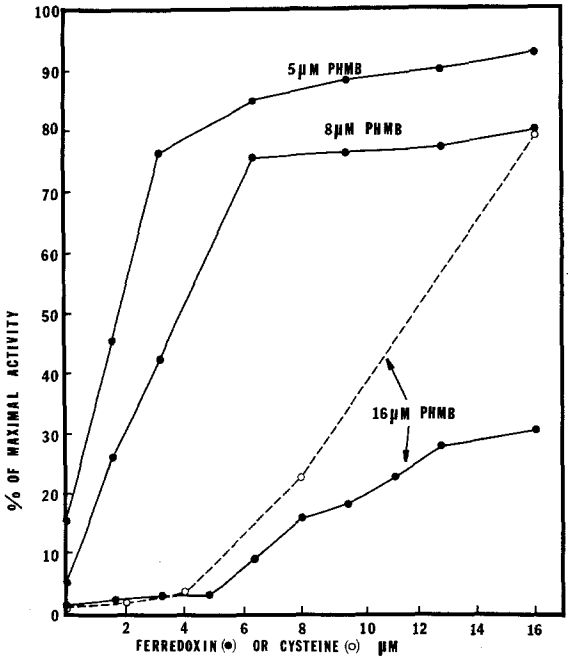

Figure 2. Reversal of PHMB inhibition by ferredoxin or cysteine. All assays were carried out using standard conditions with a G-200 enzyme preparation (168 μg protein/ml) having a specific activity of 2.86. The complete incubation mixture (less substrate) was incubated at 0° for 5 min with PHMB. Ferredoxin (●) or cysteine (○) were then added and 0° incubation was continued for an additional 5 min. Finally, substrate was added and the tubes were immediately transferred to the 20° water bath-shaker for the hydroxylation assay. Glutathione (not shown) was also tested and gave essentially the same results as cysteine.

pasteurianum ferredoxin in overcoming inhibition decreased with increasing PHMB concentrations.

Insensitivity of hydroxylation to superoxide dismutase, catalase and radical scavengers: Although the normal involvement of superoxide radical (O_2^-) in biological hydroxylations remains an open question, the participation of this radical in various enzymatic (9,10) and model (11,12) hydroxylation systems has been inferred, primarily from experiments showing inhibition of these hydroxylation reactions by SOD. Such inhibition would be expected if either free O_2^- itself or a derived product such as singlet oxygen (13) or hydroxyl radical served as the active species of oxygen. Since superoxide radical can be generated by the autoxidation of reduced ferredoxins (14), flavins (15) and P-450 type cytochromes (13), it also seems possible that O_2^- mediated hydroxylations can occur as side reactions in systems containing one or more of these components. We thought it prudent, therefore, to test for the possible involvement of free superoxide radical in the (ω-2) hydroxylation system. SOD, however, added at concentrations up to 1.5 μM, had no effect on hydroxylation in our system. Since hydrogen peroxide can arise from both the spontaneous and enzyme-catalyzed dismutation of O_2^- (12,16), the effect of added catalase was also examined. At lower levels (25-100 Sigma units) catalase had no effect while at the highest level tested (250 Sigma units) it was slightly stimulatory. Finally, various hydroxyl radical scavengers (17,18) were tested in our system. At the highest concentrations tested (100 mM) neither ethanol nor manitol had any effect while formate inhibited slightly and benzoate caused a 75-80% inhibition of palmitate hydroxylation.

DISCUSSION

Although the unequivocal proof of P-450 involvement in the (ω-2) hydroxylation system must await the spectral analysis of a more purified preparation, the marked sensitivity of the system to carbon monoxide lends strong support to this supposition. The ratio of CO to O_2 (0.25) required to cause a 50% inhibition of hydroxylation in our system (Fig. 1) is in good agreement with the CO

to O_2 ratios (0.3 - 0.5) necessary to similarly inhibit monooxygenase preparations from the yeasts Torulopsis sp. (19) and Candida tropicalis (20,21). Both yeast systems contain cytochrome P-450 and catalyze ω or (ω-1) hydroxylation of fatty acids and alkanes. Furthermore, neither the yeast nor the B. megaterium hydroxylases are strongly inhibited by azide or cyanide, two characteristic inhibitors of CO-sensitive systems involving cytochrome oxidase (22).

The strong inhibition of (ω-2) hydroxylation by PHMB was not an unexpected result since both ferredoxin and cytochrome P-450 are susceptible to this reagent (23,24). PHMB inhibition (50% at 35-50 μM PHMB) of CO-sensitive hydroxylating systems was reported by Boyd et al. (25) with a rat liver cholesterol-7-α-hydroxylase and by Ichihara et al. (4) with an ω-hydroxylation system from porcine kidney-cortex microsomes. Both of these systems contained an iron-sulfur protein in addition to the CO-sensitive component but the specific site of PHMB inhibition was not shown. Orrenius and Ernster (26) report studies by themselves and others that suggest the sensitivity of P-450 monooxygenase systems to Hg-compounds can be ascribed to a component with essential SH-groups (usually an iron-sulfur protein) which links the flavoprotein reductase and P-450. Our evidence suggests that at low concentrations of PHMB, the ferredoxin-replaceable component in our system is the primary site of inhibition while at higher PHMB levels, the P-450 component may also be involved. We base this hypothesis on the fact that ferredoxin, cysteine or glutathione are equally effective in reversing inhibition at low PHMB levels but, as the concentration of PHMB increases, ferredoxin exhibits a biphasic reactivation curve and becomes increasingly ineffective relative to either cysteine or glutathione. Yu and Gunsalus (24) have shown that inactive cytochrome formed by treatment of $P\text{-}450_{cam}$ with mercurials can be reconverted to the catalytically active form by treatment with cysteine. In our system, it may be that ferredoxin is active only in substituting for the endogenous ferredoxin-replaceable component but (for steric reasons, perhaps) cannot effectively reverse PHMB inhibition of the P-450 component.

Finally, our results with SOD, catalase and radical scavengers rule out the involvement, in free solution, of various free-radical forms of oxygen in the (ω-2) hydroxylation reaction. Sligar et al. (13) have shown that autoxidation of ferrous cytochrome P-450s can lead to O_2^- production. Spontaneous (but not SOD-catalyzed) dismutation of O_2^- can then yield singlet oxygen (13, 16,27). Furthermore, H_2O_2, a second dismutation product can in turn generate ·OH by reaction with addition O_2^- (12). In our system, the participation of free O_2^- or H_2O_2 were ruled out by the failure of either SOD or catalase to inhibit. Neither azide nor cysteine, both singlet oxygen quenches, affected hydroxylation nor did various ·OH scavengers when used at concentrations which normally inhibit systems involving this species (18). The relative insensitivity of our hydroxylase to oxygen radical scavengers and inhibitors is not unique. In this respect, it resembles the camphor hydroxylase system from Pseudomonas putida (8) which also utilizes P-450 and an iron-sulfur protein. Gunsalus et al. suggest that the close-pocket arrangement of substrate and active site in the P. putida system facilitates rapid catalysis of any unstable superoxide anion intermediates without releasing them into the medium. The limited evidence presented here and in another paper (2) suggests a similar situation in our system.

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