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REACTIVATION STUDIES ON PUTIDAMONOOXIN - THE MONOOXYGENASE OF A 4-METHOXYBENZOATE O-DEMETHYLASE FROM PSEUDOMONAS PUTIDA

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SUMMARY: Reduced putidamonooxin from a stock solution loses about 63 % of its activity within seconds when exposed to oxidizing conditions. This inactivation is prevented by the presence of substrate. Strongly inactivated putidamonooxin is reactivated, from 37 % to 66 % of its original activity, when preincubated with ferrous ions. The presence of thiol compounds in addition to ferrous ions leads to the enzyme's complete reactivation. From these results the following conclusions are drawn: The reactivation of putidamonooxin (i) depends on Fe (II) ions and (ii) involves a sulfhydryl group.
In the absence of the additional ferrous ion, reduced putidamonooxin is only partially oxidized by dioxygen. This finding indicates that the additional iron ion, possibly tightly bound to a mercaptide group, functions both as the dioxygen binding site and as the mediator of electron flow from an iron-sulfur centre to dioxygen.

INTRODUCTION: A 4-methoxybenzoate O-demethylase was isolated from Pseudomonas putida and was separated into two components: the oligomeric monooxygenase (EPR at $\bar{g}$ = 1.88), named putidamonooxin (M_r = 126 000); and the NADH-putidamonooxin oxidoreductase, a conjugated iron-sulfur protein (EPR at $\bar{g} \geqslant 1.95$) with FMN as an additional prosthetic group (1-4).
Putidamonooxin is very probably a trimer consisting of identical subunits as suggested by identical molecular weights and N- and C-terminal amino acids (5, 6). Both components, putidamonooxin and the reductase, are highly unstable in the presence of dioxygen.
Reduced putidamonooxin, however, is stable for several months when stored at 4^{o} C under anaerobic conditions in the presence of 0.3 mM sodium dithionite and 1 mM dithioerythritol.
The reductase is stable in solution for some weeks when stored at -20^{o} C under anaerobic conditions in the presence of NADH, or for several months when freeze-dried (3,7).

Abbreviations: EPR, electron paramagnetic resonance; $\bar{g}$, mean g-value = $\sqrt{(g_x + g_y + g_z)}/3$; PMO, putidamonooxin; PMO^r, reduced putidamonooxin from the stock solution; PMO^o, putidamonooxin oxidized in the absence of substrate; S, the substrate 4-methoxybenzoate; PMO^{oS}, putidamonooxin oxidized in the presence of S; RM, reactivating mixture.

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The iron and sulfur contents of putidamonooxin after the removal of any stabilizing agents by filtration on Sephadex G-25 have been evaluated. Iron and acid-labile sulfide were present in equimolar amounts. No other transition metal ions have been found (3,5).

The reconstituted 4-methoxybenzoate O-demethylase is strongly inhibited by sulfhydryl modifying reagents. Its inhibition by p-chloromercuribenzoate can be reversed by the addition of reduced glutathione, indicating that a sulfhydryl group is necessary for catalytic activity (8, 9).

We have investigated whether the extreme instability of putidamonooxin in the absence of substrate is caused by oxidation of a sulfhydryl group, or by oxidative destruction of the iron-sulfur clusters (2Fe-2S), or by loss of a transition metal ion.

MATERIALS AND METHODS: NADH (Grade I) was obtained from Boehringer (Mannheim). 3-Nitro-4-methoxybenzoate was prepared according to the procedure described by Froelicher et al. (10). All other chemicals and biochemicals were reagent grade and were obtained from the usual commercial sources.

The two components of the 4-methoxybenzoate O-demethylase were purified as described previously (3,9). The protein contents were determined according to the method of Lowry et al. (11), with bovine serum albumin and desalted and freeze-dried putidamonooxin (PMO) used as standards. All the experiments were carried out in 50 mM potassium phosphate buffer, pH 8.0, at 30° C in 1-cm semimicro cuvettes held in a temperature-controlled cell compartment fitted in an Aminco DW-2 spectrophotometer. The results are expressed as mean values ± S.E.M.; n = number of experiments. The mean values of the data for each set of experiments were compared and the significance of the differences was evaluated by means of Student's t-test.

Enzyme assay: The usual assay mixture contained 4 mM 3-nitro-4-methoxybenzoate, 0.3 mM NADH, and 22 µg PMO, in a final volume of 0.5 ml. The reaction was started by the addition of 15 µg of the reductase. When the direct effect of iron ($FeSO_4 \cdot 7\ H_2O$) or of EDTA on PMO was studied, these reagents were added to the assay mixture so as to give 0.25 mM and 0.5 mM concentrations, respectively. When the experimental enzyme sample was preincubated with 4-methoxybenzoate, causing a small amount of that substrate to be introduced into the assay mixture, we introduced the same amount of the substrate into the corresponding control assay mixtures.

The formation of 3-nitro-4-hydroxybenzoate was monitored by the increase in the difference between absorbances at 415 and at 475 nm in the dual-wavelength mode, as previously described (8). In the experiments the substrate 3-nitro-4-methoxybenzoate was always added to the assay system before the PMO, unless otherwise specified.

Procedure for rapid oxidation of putidamonooxin: The reducing agents (0.3 mM sodium dithionite and 1 mM dithioerythritol) were removed from the stock solution of PMO (resulting in the simultaneous oxidation of the enzyme) as follows. Sephadex G-25 beads (fine) equilibrated with 50 mM potassium phosphate buffer, pH 8.0, in the presence or the absence of 1 mM 4-methoxybenzoate were packed into chromatography columns (0.7 x 2.5 cm) which were fitted into centrifuge tubes of an Eppendorf 3200 centrifuge and were then centrifuged (3-10 s at 8000 x g) to remove the excluded buffer volume. Then 100-150 µl of the stock solution of reduced putidamonooxin (PMO^r) was applied and passed by centrifugation (3-10 s at 8000 x g) through the columns into the centrifuge tubes. This procedure prevents any dilution of the applied enzyme.

Reactivation procedure: PMO^r from the stock solution and PMO oxidized in the absence or in the presence of 4-methoxybenzoate (PMO^o and PMO^{oS}, respectively) were preincubated in ice under N_2 atmosphere for 15 or 60 min as summarized in Table 1 and then were assayed. The protein concentrations of the preincubated enzyme samples were 4.1 - 9.8 mg x ml^{-1} in the final preincubation volume. For the reactivation experiments, 4-methoxybenzoate (when it was used) was added to the enzyme samples 1 to 3 min before the other reagents were added, except when it was already present as a result of the gel filtration (PMO^{oS}).

RESULTS: When the PMO^r from the stock solution was added to the usual assay mixture before the substrate 3-nitro-4-methoxybenzoate, 17 ± 1,6 % (n=22) of the O-demethylation activity found in the control experiments was immediately

Table 1: Effect of various preincubation conditions on the activity of putidamonooxin.

Experiment no.	Preincubation mixture and length of preincubation	Mean activity (%) ± S.E.M.
1	PMO^r ^{xx}	100
2	PMO^r + EDTA ^{xx}	57 ± 1.97 (19)
3	PMO^r + S + EDTA ^{xx}	70 ± 3.05 (16)
4†	PMO^o + RM + EDTA ^{xx} + Fe (II) ^{xx}	96 ± 6.78 (28)
5	PMO^o + RM + EDTA ^{xx} + Fe (II) ^{xx}	100
6	PMO^o ^{x} or ^{xx}	37 ± 2.68 (28)
7	PMO^{oS} ^{xx}	96 ± 1.81 (11)
8	PMO^{oS} + EDTA ^{xx}	99 ± 2.20 (9)
9	PMO^o + Fe (II) ^{x}	66 ± 2.20 (24)
10	PMO^o + S + Fe (II) ^{x}	56 ± 2.76 (24)
11	PMO^o + p-CMB ^{x} + Fe (II) ^{xx}	35 ± 2.47 (10)
12	PMO^o + RM + EDTA ^{xx}	21 ± 2.52 (18)
13	PMO^o + S + RM + EDTA ^{xx}	46 ± 4.54 (15)
14	PMO^o + Fe (II) ^{xx} + RM ^{xx}	88 ± 2.73 (16)
15	PMO^o + p-CMB ^{x} + Fe (II) ^{xx} + RM ^{xx}	84 ± 2.28 (10)
16	PMO^o + S + RM + EDTA ^{xx} + Fe (II) ^{xx}	91 ± 1.72 (17)

^{x}, ^{xx}: Incubation for 15 (^{x}) or 60 (^{xx}) min before the next step (the addition of another substance, or the assay).

† : For experiments 6-16, the individual results of this experiment (no. 4) in each case were taken as 100 % in order to eliminate the effect of variations between different enzyme preparations.

The number of experiments is given in parentheses. S = substrate (10 mM 4-methoxybenzoate); p-CMB = 0.1 mM p-chloromercuribenzoate; RM = reactivating mixture (100 mM 2-mercaptoethanol, 16 mM dithioerythritol, and 3.8 mM sodium dithionite); EDTA was 1 mM; Fe (II) was 2 mM. 100 % corresponds to a mean specific activity in the 3-nitro-4-methoxybenzoate O-demethylation assay of 490 mU x (mg PMO)$^{-1}$. For additional details, see Methods and Results.

lost, whether EDTA was present or not. Furthermore, the oxidation of PMO^r by gel filtration led in the absence of the substrate 4-methoxybenzoate to a mean loss of 63 % of the enzyme's activity (Table 1, experiment no. 6). Surprisingly, this effect of oxidation was almost completely prevented when the substrate had been present in the equilibration buffer of the Sephadex G-25 gel (Table 1, exp. no.7).

Spectroscopic studies (EPR and absorbance) revealed that in the absence of substrate the iron-sulfur centres of PMO were not significantly destroyed during the first 2 h after the oxidation of the enzyme. Destruction of these centres are therefore not the reason for the enzyme's rapid inactivation. To find out the reason, we performed the following experiments.

PMO^r, oxidized and strongly inactivated by the gel filtration procedure in the absence of 4-methoxybenzoate (PMO^o) was preincubated in the standard assay mixture (22 µg PMO^o x 0.5 ml^{-1}) for 1 or 2 min with ferrous ions in the presence or in the absence of 3-nitro-4-methoxybenzoate before the addition of reductase started the reaction. There was no reactivation although both benzene and pyrazone dioxygenases are reactivated under these conditions (12, 13, 14). Subsequent reactivation experiments were therefore performed on concentrated enzyme solutions containing 4.1 - 9.8 mg protein x ml^{-1}. The following results, summarized in Table 1 and Figure 1, were obtained with these preincubated enzyme samples.

(a) Preincubation of the PMO^o with ferrous ions (exp. no 9) again resulted in 66 % of the original enzyme activity. The incorporation of Fe (II) ions into the PMO^o was confirmed and quantified by Mössbauer measurements and EPR studies (6, 15, 16, and E. Bill, F.-H. Bernhardt, and A. Trautwein, unpublished results). When the activity of the PMO^o in the 3-nitro-4-methoxybenzoate O-demethylation assay was used as a criterion, 1.5 mM solutions of $CoCl_2$, $MnSO_4$, Na_2MoO_4, or $(NH_4)_6\ Mo_7O_{24}$ did not mimic ferrous ions when preincubated with PMO^o at pH 8.0. The PMO^o was not reactivated by ferrous ions when the enzyme was first preincubated with p-chloromercuribenzoate (exp. no. 11). This finding led us to suggest that besides Fe (II) ions a sulfhydryl group is involved which must be protected for the enzyme's full reactivation.

To confirm this hypothesis we tried to enhance the reactivating effect of Fe (II) ions by the simultaneous addition of either thiol compounds such as 2-mercaptoethanol (100 mM) or dithioerythritol (16 mM) or of sodium dithionite (3.8 mM). Indeed, each of the three reagents conferred additional reactivation. The best results, however, were obtained when the three compounds were combined as a reactivating mixture (RM):

(b) The addition of either the RM to the enzyme sample preincubated with ferrous ions, or of ferrous ions to the enzyme preincubated with the RM in the presence of EDTA, followed by prolonged preincubation, further reactivated the PMO^o

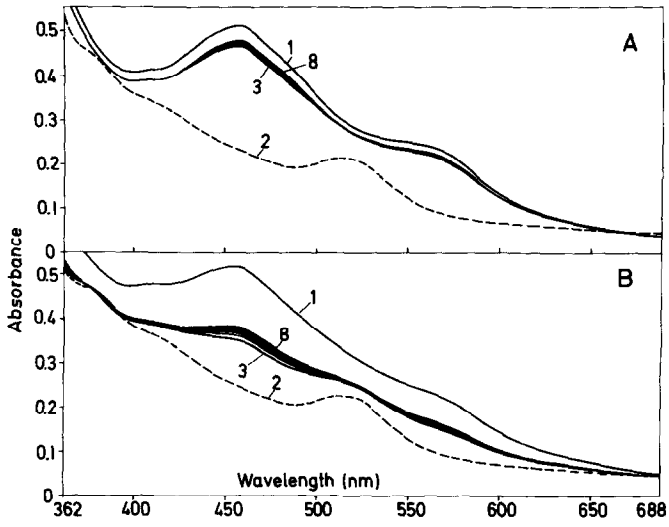

Fig. 1. Effects of ferrous ions on the autoxidation of putidamonooxin (absolute spectrum measurements).
A, PMO^{r} from the stock solution, preincubated under reducing conditions with 1 mM $FeSO_4$ for 30 to 60 min before being oxidized by gel filtration in the presence of substrate.
B, PMO^{r} from the stock solution directly oxidized by gel filtration in the absence of substrate.
The samples contained 2.55 mg PMO, pretreated as described above; 3 mM 4-methoxybenzoate; and 650 U of catalase, in 0.5 ml 50 mM potassium phosphate buffer, pH 8.0. (1) PMO^{os}; (2) PMO^{rs}, obtained from (1) by titration with minute amounts of sodium dithionite dissolved in 1 M Tris-HCl buffer, pH 8.0; (3-8) system represented in line 2, reoxidized by the rapid addition of 0.6 mM H_2O_2 to the dithionite-reduced (line 2) PMO^{rs}, after times ranging from 1 s (line 3) to approximately 30 min (line 8). Incubation at 30° C.

(means 88 % and 100 % of the original activity, respectively; Table 1, exp. nos. 14, 4 and 5).

The addition of the RM to the enzyme sample preincubated with p-chloromercuribenzoate (exp. no. 11) followed by more preincubation (exp. no 15) led to a reactivation of the enzyme to nearly the level in the control experiment (exp. no. 14).

(c) The presence of the substrate 4-methoxybenzoate influenced the inactivation and reactivation process as follows. Preincubation of the PMO^{r} with EDTA inactivated on the average 43 % of the PMO^{r} (exp. no. 2). The addition of the substrate protected the reduced enzyme against the chelator in that the mean inactivation of the PMO^{r} was now 13 % less (exp. no. 3; $p < 0.005$) and the PMO^{os} was virtually stable in the presence of EDTA (exp. no. 8).

The reactivation of PMO^{o} by ferrous ions was significantly less in the presence of the substrate (exp. nos. 9 and 10; $p < 0.005$). The same result was obtained when the PMO^{o} was preincubated with the RM in the presence of the substrate and EDTA before the addition of the ferrous sulfate and further preincubation (exp. nos. 16 and 5; $p < 0.005$).

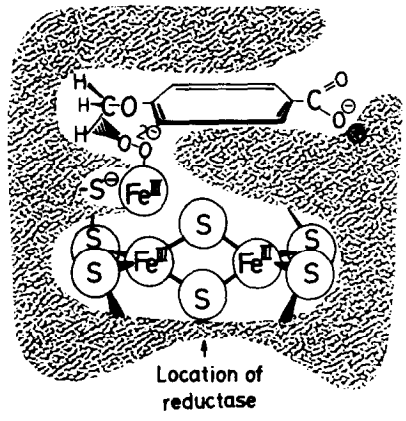

Fig. 2. Model for the active site of putidamonooxin.

(d) The experiments shown in Figs. 2A and B strongly indicate that the presence of the additional iron ion is required for the oxidation of the 2Fe-2S centres by dioxygen. The spectroscopic experiments (Fig. 2B) were performed with PMO^{O}. Line 1 shows its absorption spectrum in the presence of catalase and 4-methoxybenzoate. Titration with minute amounts of sodium dithionite in Tris-HCl buffer yielded the spectrum of the reduced enzyme (line 2). Starting with the reduced state about 40 % of the enzyme was oxidized within 1 s upon mixing with H_2O_2 (line 3). No further oxidation of the enzyme was observed during incubation for 20 to 30 min (lines 3 to 8). Note that the PMO^{O} in the presence of substrate (line 1) still contained a small amount of reduced enzyme, as can be deduced by comparing this spectrum with that of the PMO^{OS} from the experiments reported in Fig. 2A.

For Fig. 2A we used the same experimental procedure and the same assay mixture; but in this case, the PMO^{I} was preincubated with ferrous sulfate under reducing conditions before being oxidized by gel filtration in the presence of substrate, which led to PMO^{OS}. Note that when this enzyme preparation was used, the dithionite-reduced PMO^{OS} (Fig. 2A, line 2) was oxidized almost completely within 1 s by the addition of H_2O_2 (line 3).

The fully oxidized state was regained in both experiments (Fig. 2A, B) by the addition of 40 μM ferricyanide.

Treatment of PMO^{I} with 1 mM EDTA or 5 mM KCN (pH 8.0) before the gel filtration in the absence of substrate led to results similar to those summarized in Fig. 2B.

DISCUSSION: On the basis of our results, a model for the active site of putidamonooxin is developed (Fig. 2). Spectroscopic (Mössbauer, EPR and optical absorbance) studies revealed that the active site(s) of putidamonooxin con-

tain(s) besides the 2Fe-2S centre (15, 17) an additional iron (Fe^{2+}), which most probably is bound by a mercaptide group. From our investigations we conclude that this additional iron functions both as the dioxygen binding site and as the mediator of electron flow from an iron-sulfur centre to dioxygen. In the trivalent state this additional iron is bound to the active site of putidamonooxin only in presence of substrate. From results reported previously, it has been suggested that $[FeO_2]^{\oplus}$ initiates the oxygenation of the substrate or forms H_2O_2 when inactivated by protonation under uncoupling conditions (6, 18, 19). In ^2H_2O the active oxygen species is partially reduced to water when 4-trifluoromethylbenzoate is used as a completely uncoupling substrate (16). The findings of the Mössbauer studies indicate that the dioxygen activation is achieved by the uptake of one electron from the reduced iron-sulfur centre and of one electron from the reduced additional iron (15).

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