Covalently Bound FAD in d-6-Hydroxynicotine Oxidase

IMMUNOLOGICAL STUDIES ON d- AND L-6-HYDROXYNICOTINE OXIDASE: EVIDENCE FOR A d-ENZYME PRECURSOR*

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Antisera prepared against both enantozyymes, d- and L-6-hydroxynicotine oxidase, formed precipitins in double diffusion tests with their respective antigen only. A mixture of the two antisera caused spur formation of the two precipitin lines obtained with the pure enzymes. Antiserum to L-apoprotein reacted with native L-enzyme and L-apoprotein but not with the d-specific enzyme. d-6-hydroxynicotine oxidase activity was inhibited by the anti-d-antiserum, leaving the L-enzyme fully active, while anti-L-antiserum inhibited the L- but not the d-specific activity.

The delayed induction of d-6-hydroxynicotine oxidase as compared to the other activities of the nicotine-degrading sequence and the differential immunchemical behavior of the enantozyymes allowed the search for a d-enzyme precursor. In cells harvested 3 hours after the addition of dL-nicotine, the L-enzyme activity was present, whereas no d-enzyme activity could be detected. However, an extract of these cells did form an immunoprecipitin line with anti-d-antiserum.

L-6-Hydroxynicotine oxidase, but no d-6-hydroxynicotine oxidase activity, could also be induced in Arthrobacter oxidans grown in a medium with a high glucose content and dL-nicotine as the sole nitrogen source. An extract of these L-induced cells produced the specific immunoprecipitation with anti-d-antiserum. A pulse-chase experiment with cells grown first on glucose and dL-nicotine in the presence of ["Clleucine and then in an unlabeled medium which induces d-6-hydroxynicotine oxidase activity resulted in a radioactive d-enzyme-immunoprecipitin line.

From these experiments it is concluded that a precursor of the active d-enzyme is induced simultaneously with the other nicotine-degrading enzymes.

In Arthrobacter oxidans dL-nicotine induces both a d- and a L-6-hydroxynicotine oxidase (1, 2). These “enantozyymes,” a term previously proposed (3) for specific enantiomeric enzymes, show different induction kinetics (4, 5) the d-specific activity appearing much later than the L enzyme1 (5). Molecular weight, subunit structure, reaction mechanism, specificity, and reactivity toward artificial electron acceptors of both purified enzymes have been established (3). The most remarkable difference between these enzymes is their coenzyme binding. L-6-Hydroxynicotine oxidase contains FAD reversibly attached, whereas in the d-specific enzyme the FAD is covalently bound to the N-3 of a histidyl residue of the apoprotein via the 8α-methylene group of the flavin nucleus (6). This communication deals with the antigenic behavior of the enantozyymes. The immunological evidence for the presence of a d-enzyme precursor in cells lacking the active d-enzyme suggests that the retarded appearance of d-6-hydroxynicotine oxidase activity is due to the process of covalent FAD binding.

EXPERIMENTAL PROCEDURES

Homogeneous d-6 hydroxynicotine oxidase from Arthrobacter oxidans was prepared as described previously (2). Pure L-6-hydroxynicotine oxidase was obtained during the same procedure; the fractions of DEAE-cellulose chromatography containing L-enzyme activity were further purified by preparative disc electrophoresis (pH 10) and finally by gel filtration on Sephadex G-200 superfine. L-apo-enzyme was prepared by the procedure of Warburg and Christian (7). D-Amino acid oxidase (EC 1.4.3.3) was purchased from Boehringer Mannheim GmbH (Mannheim, Germany).

Antisera against both the d- and the L-enzyme and the L-apo-enzyme were induced in rabbits (hybrid of white Vienna multiplied Alasca). Enzyme (1.5 ml) and 2% Al(OH)3 (1.5 ml) were kept at 4°C for at least 1 hour; Freund’s complete adjuvants (3.0 ml) were added and the mixture was emulsified. Two milliliters of each of the emulsion, containing 0.8 mg of enzyme, were injected into the rabbit intracutaneously and into the footpads. A second injection of 2 mg of enzyme was administered subcutaneously 6 to 10 weeks later. Each rabbit was bled at 10-day intervals, beginning 10 days after the second injection. Antisera of the
first bleeding were used for the experiments.

Immunodiffusion experiments were conducted by the Ouchterlony double-diffusion technique (8) on microscope slides coated with 1% agarose gel (9). Antiserum (10 µl) were added to the center wells and antigens (10 µl) to the peripheral wells. Precipitin lines developed within 2 days at 4°. The gels were soaked in 0.5% NaCl solution for 2 days (several changes), dried at 37° overnight, stained with Amido black (0.5%), destained in methanol/acetic acid (9/1), and photographed.

Experiments to determine the inhibition of enzyme activity were performed in tapered centrifuge tubes. A crude extract obtained by sonification of fully induced cells in 10 mM phosphate buffer, pH 7.5, was incubated overnight at 25° with various amounts of antiserum and 0.9% NaCl in a total volume of 0.3 ml. After centrifugation, aliquots of sonification of fully induced cells in 10 mM phosphate buffer, pH 7.5, performed in tapered centrifuge tubes. A crude extract obtained by sonification of fully induced cells in 10 mM phosphate buffer, pH 7.5, was incubated overnight at 25° with various amounts of antiserum and 0.9% NaCl in a total volume of 0.3 ml. After centrifugation, aliquots were removed for the assays of D- and L-6-hydroxynicotine oxidase activity (3).

"3-Hour induced cells" containing only L-enzyme activity were grown in an inducing medium consisting of 3.06 g of KH₂PO₄, 9.85 g of Na₂HPO₄, 12H₂O, 0.5 g of yeast extract (Difco), 2.0 g of (NH₄)₂SO₄, 50 ml of trace salt solution (10), 2.5 g of Na₂citrate 2H₂O, and 0.5 ml of dl-nicotine/liter. A 30% inoculum of glycerol-grown cells (0.2% glycerol instead of Na₂citrate and DL-nicotine) was used. After 3 hours, when the L activity was already induced but almost no D activity was present, the cells were harvested and stored at −30°. Small quantities of cell-free extract were obtained by sonifying (Branson Sonifier J17TV, scale adjustment, 1) 5 ml of resuspended cells (A/ml at 691 nm = 50 to 60) for 5 min and centrifuging. L-Induced cells containing only L-enzyme but no D-enzyme activity were grown in a glucose medium containing 3.06 g of KH₂PO₄, 9.85 g of Na₂HPO₄, 12H₂O, 0.5 g of yeast extract, 50 ml of trace salt solution, 5 g of glucose and 2.0 ml of dl-nicotine/liter.

The pulse-chase experiment was conducted as follows: 50 ml of glucose medium were inoculated with 5% glycerol-grown cells, supplemented 5 hours later with 12.5 µCi of L-[¹⁴C]leucine (348 mCi/mmol) and aerated overnight. The cells then were transferred into a glucose medium without radioactive to exhaust the cells of endogenous [¹⁴C]leucine during further growth. Twenty-five milliliters of the culture were centrifuged and stored ("labeled L-induced cells"). The remaining 25 ml were centrifuged under sterile conditions, transferred into 60 ml of inducing medium (0.2% titrate/0.05% DL-nicotine/liter) and aeration overnight. The cells then were harvested and stored ("labeled L-induced cells"). The opposite result was obtained by incubating the extract with anti-D-antiserum (Fig. 2B). In a crude extract, the quantity of the respective antiserum required to reach 50% inhibition of activity was proportional to the amount of enzyme protein (Fig. 3).

**RESULTS**

**Immunoprecipitation of D- and L-6-Hydroxynicotine Oxidase by Respective Antiserum**—In an immunodiffusion experiment, anti-D-antiserum reacted only with pure D-enzyme and with an extract of fully induced cells containing L and D activity (Fig. 1A). No precipitin line could be detected with pure L-enzyme, even at higher concentrations, and with L-apoprotein. Anti-L-antiserum formed a precipitate with pure L-enzyme, fully induced cell extract, and L-apoprotein (Fig. 1B). D-Enzyme was not precipitated by anti-L-antiserum; D-amino acid oxidase, another FAD-containing protein, reacted with neither of the two antisera. With a mixture of the two antisera in the center well spur formation of the precipitin lines of the pure enantiomorphs was seen; the extract of fully induced cells elicited two precipitation lines (Fig. 1D). Antiserum against the L-apoenzyme produced lines of identity with L-apoenzyme, pure L-enzyme, and with an extract of induced cells, but no precipitation occurred with D-enzyme or D-amino acid oxidase (Fig. 1C).

**Inhibition of Enzyme Activity by Antibody**—When an extract of fully induced cells was incubated overnight with different amounts of anti-D-antiserum, a corresponding loss of D activity was found in the supernatant whereas the L activity was fully retained (Fig. 2A). The opposite result was obtained by incubating the extract with anti-D-antiserum (Fig. 2B). In a crude extract, the quantity of the respective antiserum required to reach 50% inhibition of activity was proportional to the amount of enzyme protein (Fig. 3).

**Immunological Detection of D-Enzyme Precursor**—The search for a precursor protein of D-6-hydroxynicotine oxidase became feasible when the different antigenicity of the enantiomorphs was established and conditions of partial induction were developed which allow to obtain cells with L activity only. These cells were grown on an inducing medium and harvested 3 hours after inoculation; at this time the high L activity began to level off but the D activity has not yet been induced (5) (Fig. 4). An extract of these cells produced a distinct, although weak, precipitin line with anti-D-antiserum (Fig. 5A). This line was different from that obtained with the L-enzyme but appeared to be identical with the weak inner line of fully induced cells (Fig. 5A); it does not pass over into the precipitin line formed with pure D-6-hydroxynicotine oxidase (Fig. 5C). Noninduced cells did not show these two precipitin lines. Furthermore, the removal of antibodies from the anti-D-antiserum by incubation with purified D-enzyme yielded a preparation which no longer reacted with the precursor protein. The possibility that a minute amount of active D-enzyme present in the 3-hour induced cell extract could have caused the distinct, weak precipitin line, was excluded since the same amount of anti-D-antiserum does not form a detectable band with less than 100 milliunits/ml of D-enzyme (not shown). The D-specific activity of the 3-hour induced cell extract, however, was less than 10 milliunits/ml. As was expected, the anti-L-antiserum reacted with the 3-hour induced cell extract but not with an extract of noninduced cells. (Fig. 5D).

**Reaction of Extract of L-Induced Cells with Anti-D-Antiserum**—When Arthrobacter oxidans was grown on 0.5% glucose as carbon and 0.2% dl-nicotine as sole nitrogen source,
Ant1 D-antiserum (µl)

% Remaining activity

0 5 10

Anti D-antiserum (µl)

FIG. 2. Inhibition of enzyme activity by antisera. Fully induced cell extract containing (A) 2.0 µg of D-6-hydroxynicotine and (B) 5.2 µg of L-6-hydroxynicotine oxidase was incubated in 0.9% NaCl with increasing amounts of antiserum overnight at 25°C; total volume, 0.3 ml. The amounts of D- and L-6-hydroxynicotine oxidase in the extract were calculated from the known specific activities of the purified enzymes. After centrifugation, D- and L-enzyme activities were measured in the supernatant. The remaining activity as percentage of the activity without antiserum is plotted versus the amount of antiserum used.

D-enzyme activity was undetectable whereas the L-enzyme was fully induced. The same result was obtained when glucose was replaced by 0.5% glycerol or 0.5% citrate. Addition of ammonium sulfate caused both enzymes to be drastically repressed, even in the presence of D-nicotine (Table I). The immunological assay of extracts of these L-induced cells lacking D-6-hydroxynicotine oxidase activity with anti-D-antiserum resulted in precipitin lines which converged with the band of the 3-hour induced cell extract (Fig. 6B). The extracts of cells grown in the presence of (NH₄)₂SO₄ did not show this precipitin line (Fig. 6A).

Conversion of Radioactive D-Enzyme Precursor into Radioactive D-Enzyme in Vivo—In a pulse-chase experiment outlined under "Experimental Procedures," cells were grown in the L-inducing glucose medium supplemented with L-[¹⁴C]-leucine ("labeled L-induced cells"). They contained no measurable D activity. However, since L-6-hydroxynicotine oxidase is induced under these conditions, any precursor of the 6-hydroxynicotine oxidase, e.g. the D-apoprotein, when induced concomitantly ought to be ¹⁴C-labeled. After transfer of the labeled L-induced cells into the nonradioactive, fully inducing medium, which allows the synthesis of all nicotine degrading enzymes including D-6-hydroxynicotine oxidase, the latter enzyme should contain radioactivity if a labeled D-enzyme precursor was present in the labeled L-induced cells. Fig. 7 shows the photograph of an Ouchterlony immunodiffusion test.
(B) and of the X-ray film exposed to this slide (A). Both the D-enzyme precursor and the active D-enzyme which were well separated, were radioactive.

**DISCUSSION**

It was demonstrated that the enantiozymes D- and L-6-hydroxynicotine oxidase from *Arthrobacter oxidans* are immunologically different. Whether this is caused by a dissimilarity of the polypeptides per se or by a different tertiary and quaternary enzyme structure cannot be decided at this time.

Among the first four DL-nicotine degrading enzymes, the induction time constant (T) of L-6-hydroxynicotine oxidase, as well as of the stereounspecific enzymes, nicotine dehydrogenase (EC 1.5.99.4) and ketone dehydrogenase (formerly called nicotine oxidase and ketone oxidase (5)) is 1 hour whereas that of D-6-hydroxynicotine oxidase is 3 hours (6). The delay of the appearance of D activity may result from the retarded induction of an enzyme system involved in the process of covalent FAD-binding. In this case, the induction of the D-apoenzyme could still be correlated to that of the other enzymes of the sequence. In fact, extracts of L-induced cells which exhibited only L- but no D-enzyme activity contained a protein that reacted with anti-D-antiserum.

Several criteria point to this antigen as a precursor of D-6-hydroxynicotine oxidase. (a) The immunological response of the precursor protein to antibodies elicited by a purified D-6-hydroxynicotine oxidase preparation is highly specific. Anti-D-antiserum which was preincubated with an excess of the D-enzyme failed to produce the characteristic precipitin line with precursor-containing cell extracts (Fig. 5). Repressed cells which synthesize neither L- nor D-6-hydroxynicotine oxidase did not contain proteins reacting with anti-D-antiserum. (b) The precursor protein could be detected immunologically not only in fully induced *Arthrobacter oxidans* which produces both enantiozymes but also in L-induced and 3-hour induced cells which contain L- but no D-enzyme activity. (c) Cells which were grown under these conditions of partial induction in the presence of [14C]leucine produced a heavily labeled precursor protein but, as expected, no radioactivity was found in the precipitin line of added D-enzyme. When these cells were transferred to an unlabeled fully inducing medium in which D-6-hydroxynicotine oxidase is synthesized the precipitin line of the D-enzyme became strongly labeled; since *de novo* protein synthesis in this medium results in the formation of unlabeled proteins, it is concluded that the polypeptide preformed in the L-inducing medium, i.e. the precursor, was converted to the active D-enzyme.

**Table I**

**Glucose effect on induction of D- and L-6-hydroxynicotine oxidase activity**

Cells were grown in an L-inducing medium (see "Experimental Procedures") either in the presence or in the absence of 0.2% (NH₄)₂SO₄. In the respective experiments, 0.5% glycerol or 0.5% citrate instead of 0.5% glucose were used. For comparison, activities obtained on a medium with 0.2% DL-nicotine as the sole carbon source are given.

|                  | - (NH₄)₂SO₄ | + (NH₄)₂SO₄ |
|------------------|-------------|-------------|
|                  | dl-Nicotine only | + Glucose + Glycerol + Citrate | dl-Nicotine only | + Glucose + Glycerol + Citrate |
|                  | milliunits/mg | milliunits/mg | milliunits/mg | milliunits/mg |
| **D-Enzyme**     | 40.1        | 0.67        | 0.48         | 0.14        |
| **L-Enzyme**     | 684.8       | 513.6       | 257.8        | 452.5       |
These observations also indicate that the induction time constant ($T_i$) of 1 hour is the same for all 4 enzyme proteins of the nicotine degradation sequence (nicotine dehydrogenase, L- and D-6-hydroxynicotinic oxidase, ketone dehydrogenase) but that D activity shows up later because of a retarded induction of an enzyme or enzyme system involved in the conversion of the precursor to the active D-6-hydroxynicotinic oxidase; it is conceivable that this conversion consists in the covalent attachment of FAD to the D-apoprotein. The identity of the precursor protein with the apoprotein of D-6-hydroxynicotinic oxidase is subject of further investigations.

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