Biochimica et Biophysica Acta, 527 (1978) 264—271

BBA 68589

PURIFICATION OF INDOLYL-3-ALKANE α-HYDROXYLASE BY AFFINITY CHROMATOGRAPHY ON INDOLYL-AGAROSE COLUMNS

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(Received April 18th, 1978)
(Revised manuscript received July 7th, 1978)

Summary

Indolyl-3-alkane α-hydroxylase was isolated from soil isolate organism, *Pseudomonas* XA, by affinity chromatography on indolyl-agarose, using different indole derivatives (L-tryptophan, *N*-acetyl-L-tryptophan, indole-3-carboxaldehyde and 3-indole-acrylic acid). With the exception of *N*-acetyl-L-tryptophan-agarose, excellent yields were obtained. The affinity chromatography step caused a 15-fold increase in the specific activity of the enzyme. The purity of indolyl-3-alkane α-hydroxylase was comparable to the preparations obtained by conventional isolation techniques; however, it showed a 7- to 10-times higher overall yield. Affinity purified indolyl-3-alkane α-hydroxylase exhibited essentially one band in polyacrylamide gel electrophoresis and on isoelectric focusing.

Introduction

Indolyl-3-alkane α-hydroxylase, a novel bacterial hemoprotein, has been recently isolated and characterized [1]. When injected into mice it caused depletion of circulating tryptophan for prolonged periods [1] and was found to have substantial antineoplastic activity against mouse sarcome, hepatoma and carcinoma tumors [2]. This enzyme may also be useful for tritium-labeling of tryptophan residues in peptides and proteins [3]. The somewhat complicated mechanism of action of this enzyme has been recently elucidated [3—5]. Experimental data indicated that the hydroxylation at the side chain carbon adjacent to the indole ring is a stepwise process. The enzyme acts as an oxidase to produce an olefinic intermediate in which the double bond is between the indole 3 position and the adjacent side chain carbon (3-alkylidene indoline).

The subsequent, 1,4-addition of water to the 3-alkylidene indoline intermediate results in the hydroxylation of the substrate.

The present conventional isolation method for indolyl-3-alkane α-hydroxylase is time-consuming and results in a relatively low yield [1]. No competitive inhibitors of the enzyme are presently known. This paper describes the purification of indolyl-3-alkane α-hydroxylase by affinity chromatography on agarose columns substituted with the substrates L-tryptophan and *N*-acetyl-L-tryptophan; also using 3-indole acrylic acid and indole-3-carboxaldehyde, which are neither substrates nor inhibitors.

Materials and Methods

Bio-Gel A-5m agarose (100—200 mesh) (Bio-Rad Laboratories, Inc., Richmond, Calif.) was used as the matrix for affinity chromatography. CNBr and 1-6-hexane diamine were purchased from Eastman Kodak Co. (Rochester, N.Y.). 3-Indole-acrylic acid, indole-3-carboxaldehyde, 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluene-sulfonate and *N*-hydroxysuccinimide were obtained from Aldrich Chemical Co., Inc. (Milwaukee, Wisc.). L-tryptophan was purchased from General Biochemicals (Chagrin Falls, Ohio). *N*-acetyl-L-tryptophan was purchased from Sigma Chemical Co. (St. Louis, Mo.). Glutaraldehyde (electron microscopy grade) was from J.T. Baker Chemical Co. (Phillipsburg, N.J.). All other reagents used were also reagent grade.

Synthesis of amino-hexane-agarose

500 ml Agarose suspension (Bio-Gel A-5m) was washed with distilled water to remove NaN_3 and equilibrated with 0.2 M Na_2CO_3. The equilibrated gel was suspended in 1 l 0.2 M Na_2CO_3. The temperature of the gel suspension was adjusted to 15°C with crushed ice. 100 g CNBr in 100 ml dimethyl formamide were added to the gel suspension with rapid stirring, and activation was performed for 15 min. Temperature was kept at 15—20°C by adding crushed ice. pH 11.0 was maintained by the addition of 12 M NaOH.

Excess CNBr was then rapidly removed by filtration, and the gel washed successively with 500 ml distilled water (22°C), 2 l 0.15 M sodium phosphate, pH 7.3 (4°C), and again 500 ml distilled water (4°C). As much water as possible was removed from the gel by suction. The wet activated agarose was then mixed with 500 ml 10% (w/v) 1,6-hexane diamine solution (pH 10.5), and the mixture incubated for 60 min at room temperature. The solution was then adjusted to pH 9.5 with 5 M HCl and further reaction of 1,6-hexane diamine with the activated gel was carried out at 4°C for 24 h. With the use of 2 different pH values for the coupling of the ligand, advantage was taken of the rapid initial binding of 1,6-hexane diamine to agarose, combined with good stability of the formed amino hexane-agarose in the second part of the reaction procedure.

Excess 1,6-hexane diamine was removed, and the amino-hexane-agarose was washed extensively with 10 l distilled water, 5 l phosphate-buffered saline (pH 7.2) and 0.025 M sodium acetate (pH 5.5). The gel was equilibrated with 0.1 M sodium phosphate (pH 6.9) and stored at 4°C in this buffer with 0.02% NaN_3 added as a preservative. Precautions were taken to avoid the unpleasant nature of the activating compounds.

This amino-hexane-agarose was used for synthesis of all indolyl-agarose gels described below.

Synthesis of L-tryptophan-agarose

Amino-hexane-agarose (equivalent to 500 ml Bio-Gel) was washed with 0.1 M sodium phosphate buffer (pH 6.9) to remove NaN_3 and equilibrated with 2 vols. 10% (v/v) glutaraldehyde/0.1 M sodium phosphate (pH 6.9). Activation of amino-hexane-agarose was allowed to proceed for 4 h. Excess glutaraldehyde was removed by extensive washing with 6 l 0.1 M sodium phosphate (pH 6.9) and 4 l 0.1 M sodium phosphate (pH 7.5). The gel was then mixed with 1 l 5% L-tryptophan solution in the pH 7.5 buffer. L-tryptophan was solubilized by the addition of about 30% (v/v) dimethylformamide. The binding of tryptophan to activated amino-hexane-agarose was allowed to proceed for 20 h. All of the above steps were conducted at room temperature. Excess tryptophan was removed and the gel washed with 5 l 0.1 M sodium phosphate (pH 6.9). The L-tryptophan-agarose gel was stored in the same buffer at 4°C with 0.02% NaN_3.

Synthesis of N-acetyl-L-tryptophan-agarose and 3-indole-acrylic acid-agarose

The underlying principle is the formation of an intermediate ester with *N*-hydroxysuccinimide with these indole derivatives under anhydrous conditions and the ready formation of a carbamide bond between the indole compounds and amino-hexane-agarose under aqueous conditions.

5 g *N*-acetyl-L-tryptophan (approx. 20 mmol), 12 g carbodiimide (approx. 25 mmol), and 4 g *N*-hydroxysuccinimide (approx. 36 mmol) were dissolved in 100 ml H_2O-free dimethylformamide and allowed to react at room temperature for 1 week. A precipitate representing acyl urea was removed by filtering. The *N*-hydroxysuccinimide-ester was stored at 4°C in well-stoppered glass bottles prior to use. 4 g 3-indole-acrylic acid (approx. 22 mmol), 12 g carbodiimide, and 4 g *N*-hydroxysuccinimide were dissolved in 100 ml H_2O-free dimethylformamide. Reaction and storage conditions were as described above. Although the reaction between these reactants to form an intermediate ester should take place at an equimolar concentration, an excess of carbodiimide and *N*-hydroxysuccinimide was used since the chemical quality of these compounds could not be readily assessed due to possible chemical deterioration of carbodiimide and the hydroscopy of *N*-hydroxysuccinimide. About 1 g equivalent of these intermediate esters was reacted with 20 g wet amino-hexane-agarose in 20 ml 0.1 M sodium borate (pH 8.5) at room temperature for 1 h. The pH was maintained at 8.5 with 1 M NaOH. Before mixing with the intermediate esters, amino-hexane-agarose was equilibrated with the 0.1 M sodium borate buffer. The resulting *N*-acetyl-L-tryptophan and 3-indole-acrylic acid-agarose gels were washed with about 30 vols. 0.1 M sodium phosphate (pH 6.9)/30% (v/v) dimethylformamide, equilibrated with the phosphate buffer alone and stored at 4°C with NaN_3.

Synthesis of indolyl-agarose by reduced Schiff base formation (indolyl-agarose 'reduced Schiff base')

5 g Of indole-3-carboxaldehyde were dissolved in 200 ml 0.1 M Na_2CO_3/

$NaHCO_3$ (pH 9.5)/30% (v/v) dimethylformamide and mixed with 200 g agarose which had previously been equilibrated with the same dimethylformamide-containing buffer. The mixture was incubated for 4 h at room temperature with gentle stirring. 2 g $NaBH_4$ were then added to reduce the Schiff base to the secondary amine. The mixture was acidified with 0.1 M HCl to pH 5.5, excess $NaBH_4$ was removed and the gel washed with 2 l each of 0.025 M sodium acetate (pH 5.5) and 0.1 M sodium phosphate (pH 6.9). Storage conditions were as described above.

Isolation of indolyl-3-alkane α-hydroxylase

All four indolyl-agarose columns showed the same adsorption and elution charactertistics. Therefore, only one typical isolation procedure on 'indolyl'-agarose will be described.

Pseudomonas XA (ATCC No. 29574) cells were cultured and harvested as previously described [1]. Harvested cells were suspended in 2—3 vols. of 0.04 M potassium phosphate-buffer, pH 6.5, and sonically disrupted with a Bronwill Biosonik IV sonifier with three 3-min bursts. The sonicate was centrifuged 1 h at 9000 × *g*. L-tryptophan was added to the cell extract to a concentration of 0.5 mM, and the enzyme solution was heated at 55°C for 15 min with gentle agitation. After cooling to 4°C, the precipitate was removed by centrifugation. Solid ammonium sulfate (263 g/l) was added slowly to the supernatant enzyme solution while maintaining pH 6.5 by dropwise addition of ammonium hydroxide. After 30 min at 4°C, the precipitate was removed by centrifugation. Ammonium sulfate (125 g/l) was then added to the supernatant, and the resulting precipitate was collected by centrifugation, suspended in 0.02 M sodium acetate (pH 5.5) to give approx. 30 units per ml. This crude enzyme solution was then dialyzed for 48 h at 4°C against 0.02 M sodium acetate (pH 5.5) with frequent changes of the dialysis buffer. A precipitate which formed was removed by centrifugation and the supernatant (containing all the indolyl-3-alkane α-hydroxylase activity) was applied to an indolyl-agarose column previously equilibrated with the pH 5.5 acetate buffer. Indolyl-3-alkane α-hydroxylase was allowed to enter the column until enzyme activity started to appear in the eluate. The column was then washed with 0.02 M sodium acetate (pH 5.5) until the absorbance at 280 nm was less than 0.03 (1-cm cell). The column was eluted with 0.2 M sodium acetate (pH 5.5) buffer. The diluted enzyme was precipitated by the addition of 390 mg $(NH_4)_2SO_4$ per ml solution. After centrifugation, the precipitate was dissolved in 20 mM potassium phosphate-buffer (pH 6.5) to give about 100 U/ml, and dialyzed for 24 h against the same buffer with frequent changes of the dialysis buffer. The dialyzed samples were then frozen at −80°C.

In the instances where impurities were present, a gel filtration step was added after affinity chromatography. 5 ml Of the concentrated indolyl-3-alkane α-hydroxylase was passed over a Bio-Gel A-5m column (2.5 × 100 cm) equilibrated with 0.025 M sodium acetate (pH 5.5) with a flow rate of about 50 ml per h. The diluted enzyme was again concentrated by $(NH_4)_2SO_4$ precipitation and dialyzed as described above. All purification procedures including $(NH_4)_2SO_4$ precipitation were carried out at 4°C.

Enzyme assays

The following assay procedures were employed for determination of enzyme activity.

(a) Tryptophan Color Reagent C assay. 10 μl of enzyme solution (diluted with cold 0.04 M potassium phosphate buffer (pH 6.7) to approx. 1 U/ml) was added to 0.5 ml of 0.1 M sodium acetate buffer (pH 5.5) containing 0.6% sodium dodecyl sulfate. The reaction was initiated by adding 10 μl of L-tryptophan solution (40 mM). Incubations were conducted for 15 min at 37°C on a New Brunswick Aquatherm shaker at 200 rpm. The reaction was stopped by heating the tubes for 2 min at 100°C. The color. which measures the concentration of tryptophan, was developed by adding 4 ml of Color Reagent C [6] and heating 15 min at 60°C. After cooling, the absorbance was measured at 575 nm.

One unit of activity is defined as that amount of enzyme which catalyzes the breakdown of 1 μmol of L-tryptophan/min under the conditions of this assay.

(b) N-acetyltryptophanamide assay. The assay is based on the observation that the reaction product formed from *N*-acetyltryptophanamide by indolyl-3-alkane α-hydroxylase shows an absorption peak at 333 nm with a molar absorption coefficient $\epsilon^{H_2O}_{333nm} = 19\,820$ [5]. 10 μl indolyl-3-alkane α-hydroxylase (1 U/ml, as determined by the Reagent C color assay) was added to 1 ml 1 mM *N*-acetyltryptophanamide/0.1 M sodium phosphate (pH 7.0). The mixture was incubated at 30°C and absorbance readings (1-cm cell) were made every minute for 10 min. The increase in the absorbance at 333 nm was correlated with the reagent C assay and converted to enzyme units/ml. Protein was determined by the method of Lowry et al. [7].

Results

Fig. 1 shows a typical elution profile of indolyl-3-alkane α-hydroxylase from 3-indole-acrylic acid-agarose. All other indolyl-agarose columns described in this communication showed the same adsorption and elution characteristics. Some minor enzyme activity was eluted with 0.1 M sodium acetate (pH 5.5) but the main activity peak appeared after elution with 0.2 M sodium acetate (pH 5.5). The wash with 0.1 M sodium acetate (pH 5.5) did not remove any appreciable amount of contaminants. Therefore, the elution step with 0.2 M sodium acetate was performed immediately after the adsoprtion step. The activity profile was somewhat sharper than the protein profile, which shows some tailing. The minor enzyme activity in the tailing portion of the protein profile was therefore discarded. The final $(NH_4)_2SO_4$ step used to concentrate the diluted enzyme solution was fully quantitative. Although the specific activity of enzyme purified by different affinity gels was similar (approx. 15 U/mg protein), there were differences in the yield of recovered enzyme using different affinity columns. The yield was nearly 100% with the use of the 'non-substrate' indolyl columns. i.e., 3-indole-acrylic acid-agarose and indolyl-agarose 'Schiff-base' L-tryptophan-agarose showed a reduced yield of about 70% on the average and *N*-acetyl-L-tryptophan-agarose had an average yield of only 30%. The latter two affinity columns are representative of substrate indolyl columns. Higher molarities up to 1 M sodium acetate (pH 5.5) did not

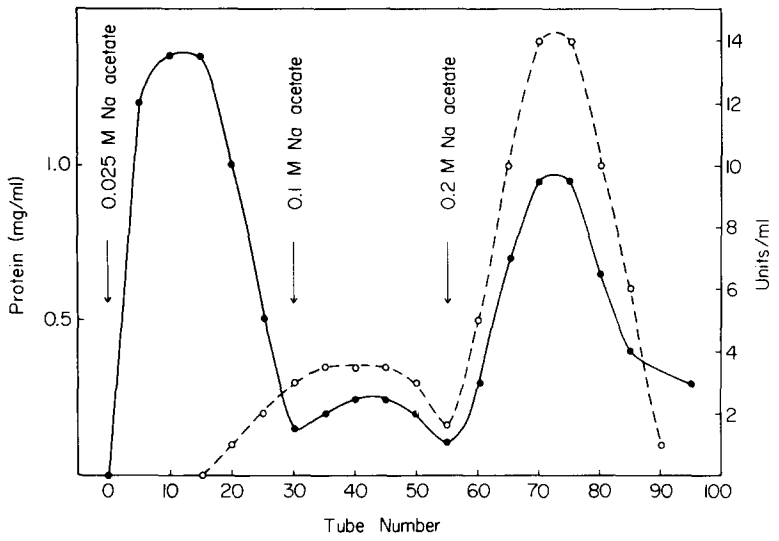

Fig. 1. Elution profile of indole-3-alkane α-hydroxylase on 3-indole-acrylic-acid-agarose. (●———●) protein, (○- - - - -○) enzyme activity. 60 ml Of packed 'indolyl'-agarose in Kontes glass columns (3 X 20 cm) were satured with 1800 U indole-3-alkane α-hydroxylase (5-ml fractions; flow rate approx. 120 ml/h).

improve the yield, indicating tight binding of a portion of the enzyme to the affinity columns.

The specific activity of indolyl-3-alkane α-hydroxylase increased from an average of 0.8 units of crude enzyme (after $(NH_4)_2SO_4$ precipitation)/mg protein to an average of 15 U/mg protein after affinity chromatography on 'non-substrate' indolyl-agarose columns. This specific activity value is similar to the purest preparations obtained by conventional purification procedures [1]. Fig. 2 shows polyacrylamide gel electrophoresis profiles of crude and affinity-purified enzyme. The affinity purified enzyme was essentially homogeneous by electrofocusing in ampholine polyacrylamide gel. There was a slight doublet peak visible on polyacrylamide gel electrophoresis which was not present in more crude preparations. This could represent enzyme which has been partly denatured by proteolysis. Fig. 2B represents indolyl-3-alkane α-hydroxylase

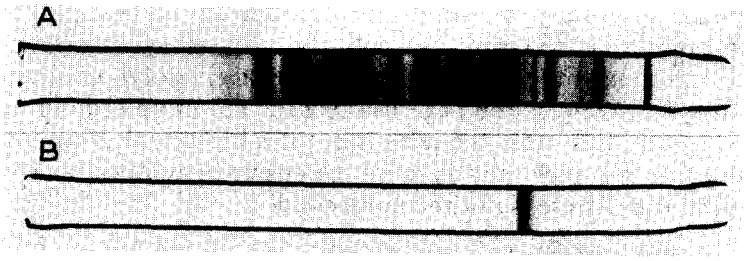

Fig. 2. Polyacrylamide gel electrophoresis of crude (A) and affinity purified enzyme (B). Approx. 5 μg pure enzyme and 100 μg crude enzyme were layered on each gel and subjected to electrophoresis in 0.05 M Tris/glycine buffer (pH 8.4). The stacking gel was 3% acrylamide in 0.25 M Tris/HCl buffer (pH 6.8) and the separation gel was 7.5% acrylamide in 0.03 M Tris-HCl buffer (pH 8.4). Protein was stained by Coomassie Brilliant Blue R-250 (Bio-Rad) in trichloroacetic acid.

purified by 3-indole-acrylic acid agarose. Indolyl agarose 'Schiff base' columns yielded similarly pure enzyme. Affinity-chromatography-purified enzyme was stable in 20 mM potassium phosphate (pH 6.5) at 4°C for at least 2 weeks.

The binding capacity of L-tryptophan-agarose and the 'non-substrate' indolyl agarose was between 30 and 60 units indolyl-3-alkane α-hydroxylase per ml packed gel under the conditions described above. The elution pattern of the enzyme was the same with different batches of affinity gel. However, enzyme preparations obtained from L-tryptophan-agarose, particularly with high binding capacity (60 U/ml), still exhibited several contaminants on polyacrylamide gel electrophoresis. A gel filtration step on Bio-Gel A-5m removed the non-active protein and increased the specific activity to 15 U/mg protein. The enzyme activity was eluted first and was well separated from small molecular weight contaminants. Enzyme activity was fully recovered after the gel filtration step.

Discussion

In the absence of any known competitive inhibitor for indolyl-3-alkane α-hydroxylase, we initially intended to synthesize affinity columns with the substrates, L-tryptophan and *N*-acetyl-L-tryptophan, to take advantage of specific enzyme-substrate interactions. The fact that we could use these 'substrate columns' several times without any loss of capacity or a change in the elution profile suggested that the enzyme binds reversibly primarily to the indole ring of the insolubilized substrates, and that the side chain, the site of the enzymatic attack, played a minor role in the binding of indolyl-3-alkane α-hydroxylase. The identical binding and elution characteristics of the enzyme with 'non-substrate indolyl columns' also support this concept. The lower yield on the substrate indolyl columns, especially on *N*-acetyl-L-tryptophan-agarose seems to indicate, however, that a portion of indolyl-3-alkane α-hydroxylase is tightly bound to the substrate, possibly by an additional binding through the side chain. Taking into account the complexity of this enzyme-substrate interaction with the formation of several intermediates [4,5], a clear assessment of the nature of binding of indole-3-alkane α-hydroxylase to the insolubilized substrate is currently not possible. It is clear, however, that the indole portion of the substrates is crucial for the affinity purification procedure of the enzyme. The present paper offers several alternatives for the purification of indole-3-alkane α-hydroxylase describing different indolyl-agarose columns. The L-tryptophan-agarose column is the simplest and cheapest to synthesize. It exhibits, however, a lower enzyme yield. The 'non-substrate indolyl' columns (3-indole-acrylic acid-agarose) and indolyl-agarose 'Schiff' are more tedious to prepare and more expensive, but offer the advantage of a high yield. *N*-acetyl-L-tryptophan-agarose has a low yield and is more expensive to synthesize than L-tryptophan-agarose, so that its use is not recommended.

Acknowledgments

This work is supported by U.S. Public Health Service grants CA-15674, CA-18844 and CA-08748. We thank Ms. Elvira Longordo for excellent technical assistance and Dr. Henry Rosenfeld for helpful suggestions.

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