Synthesis of the Pyrrole Porphobilinogen by Sepharose-Linked δ-Aminolevulinic Acid Dehydratase

Abstract. *δ-Aminolevulinic acid dehydratase* from Rhodopseudomonas spheroides *was covalently linked to Sepharose 4B, which had been activated with cyanogen bromide. A column containing this enzyme gel readily catalyzed the synthesis of the pyrrole porphobilinogen on continuous passage of a solution of δ-aminolevulinic acid. Under the conditions of the procedures, product inhibition was minimized and a 50 to 94 percent yield was attained. A column containing about 1 milligram of enzyme was continuously operated for 27 days. Although its total activity appeared to be reduced about 30 percent at the end of this time, the bound enzyme produced approximately 200 milligrams of porphobilinogen each day, and about 5 grams of the pyrrole were isolated.*

In order to study biosynthetic mechanisms and pathways it is generally helpful to have adequate quantities of substrates available and, at times, especially helpful to have the substrates labeled in particular atoms and positions with radioactive or stable isotopes. In studies involving molecular spectroscopy it is often necessary to have the substrates contain a very high concentration of ^{13}C, ^{15}N, or ^{2}H. These requirements can be met if a synthesis is available which is experimentally convenient and gives adequate yields with respect to time spent and cost. The synthesis of the intermediate meeting the suggested specifications can be also achieved enzymically from both unlabeled and labeled substrates. However, with labeled substrates, in many known instances the yields are not sufficient, nor can compounds be isolated with the required ease.

In studies concerned with the mechanisms and pathway of synthesis of porphyrins, vitamin B_{12}, and chlorophyll, and of their intermediates, substrates beyond δ-aminolevulinic acid (*1*) are rather difficult to obtain in sufficient quantities, and it is indeed especially difficult to make these compounds contain high concentrations of stable isotopes. The advantages of studying the biosynthesis of vitamin B_{12} by nuclear magnetic resonance spectroscopy with ^{13}C has been demonstrated (*2*).

The interesting mechanism of formation of the biological functional isomer of porphyrins and related compounds from the pyrrole porphobilinogen has yet to be elucidated. The study would be greatly aided, of course, if porphobilinogen in sufficient quantities, both unlabeled and labeled in particular positions, were readily available. Porphobilinogen has been synthesized both enzymically and chemically. For the chemical approach (*3*, *4*), the procedure of Frydman *et al.* (*4*) appears to give the best yield (8 percent); yet it takes a great deal of time to obtain gram quantities. The time required to synthesize porphobilinogen, labeled in different positions, would be considerable. The enzymic method (*5*, *6*) would appear to be preferable, but it is very limited because of the occurrence of product inhibition. The enzymic synthesis appears to halt when the porphobilinogen reaches a concentration of approximately 2 m*M* (*6*). Furthermore, the isolation of the pyrrole from the protein solution is both time consuming and subject to considerable loss.

In order to take advantage of the enzymic method and yet minimize product inhibition and isolation losses, we investigated the preparation of a solid support, covalently linked derivative of δ-aminolevulinic acid dehydratase, the enzyme that catalyzes the synthesis of porphobilinogen from two molecules of δ-aminolevulinic acid (Fig. 1). We now report our study of the continuous synthesis of porphobilinogen by δ-aminolevulinic acid dehydratase that is covalently linked to Sepharose 4B. Solid support enzymes are now widely used for many biochemical processes. However, almost without exception these preparations are used in hydrolytic reactions (*7*). We now describe essentially one of the few instances of the application of an enzyme gel to a synthetic process.

δ-Aminolevulinic acid was synthesized by condensation of 2-phenyl-5-oxazolene (*8*) with carboxymethoxypropionyl chloride essentially by the method of Hearn and Wildfeuer (*9*). Sepharose 4B was activated with cyanogen bromide by the method of Porath and co-workers (*10*) and Cuatrecasas *et al.* (*11*). AG1-8X resin (100–200 mesh) in its acetate form was purchased from Bio-Rad. The porphobilinogen was determined with Ehrlich's reagent (*p*-dimethylaminobenzaldehyde) as modified by Mauzerall and Granick (*12*). The definition of a unit of activity was that of Nandi *et al.* (*13*). The δ-aminolevulinic acid dehydratase of the photosynthetic bacterium *Rhodopseudomonas spheroides* was purified by a new procedure in which affinity chromatography is used (*14*). The enzyme preparation contained usually 25 to 30 units of activity per milligram of protein (*13*) and therefore was approximately 25 percent pure, but it did not contain any uroporphyrinogen synthetase.

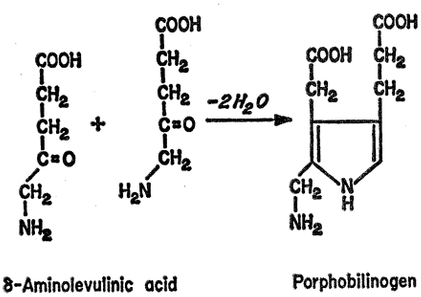

Fig. 1.

The activated Sepharose was coupled to the enzyme by the following procedure. In a typical experiment, to 70 g (wet weight) of a well-washed sample of activated Sepharose 4B, which was suspended in an equal volume of 0.03*M* potassium phosphate, *p*H 7.5, which contained β-mercaptoethanol (0.001*M*), we added 10 ml of an enzyme solution containing about 10 mg of protein whose specific activity was 25 units per milligram. The enzyme solution was previously dialyzed for 20 hours against a similar potassium phosphate buffer which also contained 5 percent glycerol. The coupling was permitted to proceed for about 20 hours at 4°C, with gentle stirring. The enzyme gel was poured onto a fritted glass funnel and washed with an equal volume of the potassium phosphate buffer. To the combined filtrate and washings, an additional 70 g of activated Sepharose was added, and the suspension was gently stirred for 3 hours at 4°C. The combined enzyme gel was washed successively with 1 liter each of the potassium phosphate buffer, 0.1*M* tris chloride, *p*H 8.4, until the washings contained no enzyme activity and finally with 0.03*M* potassium phosphate, *p*H 7.9, which contained 0.001*M* β-mercaptoethanol. The gel appeared to contain about 110 to 125 units of enzymic activity, or 1 mg of enzyme (*13*), as determined from a sample. The enzyme-gel preparation was put into a column of 25-mm inside diameter and the total volume of the bed was about 200 ml.

Solutions (0.0025*M* to 0.01*M* of δ-

aminolevulinic acid hydrochloride, previously neutralized with KOH, in 0.03*M* potassium phosphate, *p*H 7.9, containing 0.001*M* β-mercaptoethanol were passed through the enzyme-gel column (40 to 200 ml) kept at 36°C at a flow rate of about 13 to 26 ml/hour regulated by a peristaltic pump. The eluate was passed directly through a resin column of AG1-8X, in its acetate form, which retains the porphobilinogen and very little of the δ-aminolevulinic acid (*12*). The latter column contains 10 ml of resin bed for every 150 ml of anticipated eluate. Ordinarily a column (with an inside diameter of 25 mm) containing 100 to 200 ml of resin is used and is replaced by a fresh resin column when it is saturated with porphobilinogen. Analysis of samples of the eluate of the enzyme-gel column showed that the conversion of δ-aminolevulinic acid to the pyrrole porphobilinogen was 50 to 90 percent and depended on the flow rate and size of the column and concentration of δ-aminolevulinic acid. In order to isolate the porphobilinogen, the resin column was first washed with distilled water, and then treated with three to four volumes of 1*M* acetic acid and with 0.1*M* acetic acid until no more porphobilinogen was eluted. At times a portion of the porphobilinogen as the hydrate crystallizes out from the 1*M* acetic acid solution. Usually the porphobilinogen is obtained as the acetate by lyophilization of the acetic acid solution.

In a typical short experiment 334 mg (2 mmole) of δ-aminolevulinic acid hydrochloride was passed through a 40-ml enzyme-gel column and 170 mg of porphobilinogen acetate (60 percent yield) was isolated, which was essentially pure without recrystallization. Elemental analysis indicated (percent): C, 49.5; H, 6.3; N, 9.3. The calculated values for $C_{10}H_{14}O_4N_2 \cdot CH_3COOH$ are (percent): C, 50.4; H, 6.3; N, 9.8. The nuclear magnetic resonance (NMR) δ values were 2.2 ppm (3H, CH_3COOH); 2.7 ppm (4H, $-CH_2-CH_2-COOH$); 3.7 ppm (2H, $-CH_2COOH$); 4.5 ppm (2H, $-CH_2NH_2$). The porphobilinogen acetate was crystallized as the hydrate by dissolving the material in 4*M* ammonium hydroxide and then adding acetic acid to *p*H 4. Elemental analysis indicated (percent): C, 49.1; H, 6.7; N, 11.2. The calculated values for $C_{10}H_{14}O_4N_2 \cdot H_2O$ are (percent): C, 49.2; H, 6.6; N, 11.5. The NMR (*15*) spectrum of the hydrate was like the one above but no longer had the peak of the methyl group of the acetic acid at 2.2 ppm. The R_F values on cellulose-coated plaster plates were 0.52 in a solvent system of butanol, water, and acetic acid (4 : 5 : 1) and 0.43 in a solvent system of propanol, ammonia, and water (6 : 3 : 1) and were the same as those found with an authentic sample. Furthermore, the product was readily converted to uroporphyrins by warming it in 0.5*N* HCl and letting the solution stand exposed to air.

In a much larger experiment either an 0.01*M* solution of δ-aminolevulinic acid in potassium phosphate buffer was passed through a 200-ml enzyme-gel column containing approximately 110 units of enzyme activity at a flow rate of 13 ml/hour or a 0.0025*M* solution of δ-aminolevulinic acid was passed through the same column at a flow rate of 26 ml/hour. Under these conditions approximately equal amounts of porphobilinogen (200 mg) were synthesized per day, but the yield was about 50 percent under the former conditions and about 90 percent under the latter conditions. The column was operated continuously for 27 days at 36°C and 5 g of porphobilinogen was isolated. At the end of this time the total activity of the column had decreased only about 30 percent. The eluate of the resin column containing unused δ-aminolevulinic acid can be recirculated automatically through the enzyme-gel column after further addition of the substrate and potassium phosphate.

In order to minimize product inhibition on the column, both the concentration and the flow rate must be controlled. The concentration of porphobilinogen increases as the solution flows down the column, and, if it reaches inhibitory concentrations before the end of the column, the remainder of the enzyme gel will in essence not be functioning. One can avoid or minimize this effect with the use of low concentrations of substrate or by increasing the flow rate of a more concentrated solution of the substrate. Of these two procedures the percentage conversion in the former method may be higher, whereas the amount synthesized per unit time may be greater in the latter. We have usually adopted a procedure that is a compromise between achieving the highest yield and the optimum amount per unit time. It is obvious that a column that contains more enzyme units and is still of manageable size in an ordinary laboratory would produce grams of porphobilinogen per day.

The enzyme retained most of its activity for a month. At the time of the interruption of the experiment, the rate of formation of porphobilinogen was approximately the same as during the first few hours, although the total activity had fallen to about 70 percent of the initial value. In another experiment a column that was in operation for 1 week at 36°C was stored for 2 months at 4°C and still retained about 50 percent of its initial activity.

Presumably the enzyme is linked to the Sepharose via amino groups (*16*) other than the reactive ε-amino group of lysine which forms a Schiff base with the substrate (*17*). Apparently this amino group is in a cleft not readily available to react with the iminocarbonic acid formed on treatment of the Sepharose with cyanogen bromide. Since the iminocarbonic acid groups formed on reacting Sepharose with cyanogen bromide are most likely randomly distributed, it would seem reasonable to assume that, if the enzyme is active only when it retains its quaternary structure (*18*), perhaps only 1 or, at the most, 2 subunits of every active enzyme molecule are covalently linked to the Sepharose. If this is indeed so, it would appear that the binding forces among these subunits are exceedingly strong because of the remarkable stability of the enzyme under the conditions of the experiment. On the other hand, the stability of the enzyme molecule may be due to the chance covalent binding of the Sepharose of each of the subunits of the enzyme in its quaternary structure, or perhaps only a subunit is needed for enzymic activity.

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Antibodies to a Precursor of Human Collagen

Abstract. *Antiserum prepared against serum-free medium from human fibroblast cultures reacted specifically with protropocollagen, an assembled, soluble precursor of tropocollagen. The antibodies were directed to antigenic determinants in the nonhelical, amino terminal peptide extensions (propeptides). Amino acid sequences in the precursor molecule corresponding to the telopeptides and helical alpha chains of tropocollagen were not recognized by the antiserum.*

Tropocollagen, the molecule extracted from extracellular collagen fibers, is generally composed of two identical $\alpha 1$ chains and one $\alpha 2$ chain. The chains are in triple helical array except for regions about 15 amino acids long (telopeptides) at the NH_2- and COOH-terminal ends of the three chains. Antibodies to tropocollagen have been prepared in many laboratories (*1*), but conventional protocols for immunization generally fail to elicit high-titer antiserums. In most cases, the antibodies against collagen are directed against determinants in the telopeptide segments, and the helical portions of the three chains are less immunogenic.

Precursor forms of the collagen chains (procollagen; pro $\alpha 1$ and pro $\alpha 2$ chains) have been reported (*2, 3*); these chains have nonhelical, NH_2-terminal peptide extensions (propeptides) of approximately 200 amino acid residues. In human diploid fibroblasts in culture (*3*), procollagen chains are assembled intracellularly to form a molecule (protropocollagen) composed of two pro $\alpha 1$ chains and one pro $\alpha 2$ chain. This molecule is the soluble, secreted form of collagen, and is stabilized by interchain disulfide bonds between cysteine residues in the propeptides and by noncovalent interactions between the helical α chain segments. The propeptides are sequentially excised by an extracellular enzyme (or enzymes), initially generating a family of soluble intermediates and ultimately producing tropocollagen with its abbreviated NH_2-terminal telopeptides. As the latter have some immunogenic potential, we anticipated that protropocollagen with its longer nonhelical propeptides would prove to be a more potent immunogen.

Rabbits were immunized with concentrated, serum-free culture medium containing protropocollagen (*4*). When the antiserum was titrated against radioactive medium, 45 percent of the labeled protein was specifically precipitated at antibody excess (Fig. 1). To characterize the radioactive protein in the immune precipitates, washed precipitates were solubilized and subjected to electrophoresis on polyacrylamide gel containing sodium dodecyl sulfate (SDS) (*3*). Figure 2a shows the electrophoretic patterns both for medium after 24 hours of labeling with [^{3}H]-glycine and [^{3}H]proline, and for a solubilized immune precipitate from that medium run on a parallel gel. In earlier studies (*3*) peak A was identified as intact protropocollagen, and the other lettered peaks were found to be shortened pro α chains released by SDS and urea from digestion intermediates of protropocollagen. Only intact protropocollagen was precipitated, and digestion intermediates with partially excised propeptides were not recognized by the antiserum. From the electrophoretic pattern of the control medium, it can be calculated that peak A contains 45 percent of the total radioactivity in procollagen species, confirming the titration data of Fig. 1.

To demonstrate further that the antiserum was directed against protropocollagen molecules, the radioactive immune precipitates were subjected to three procedures used earlier (*3*) to characterize protropocollagen (Fig. 2b). (i) Immune precipitates were digested with purified collagenase. After digestion, 70 percent of the radioactivity was dialyzable. When the remainder was applied to gels, the protropocollagen peak was absent; this confirmed that the material in the peak is a collagenous protein. (ii) Immune precipitates were solubilized in 0.5*M* acetic acid and incubated with pepsin under

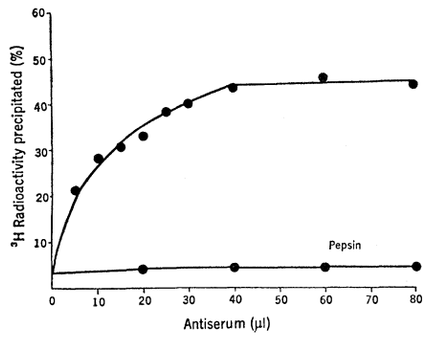

Fig. 1. Titration of antiserum against untreated and pepsin-treated radioactive medium. Test antigen was prepared by labeling confluent plates of human diploid fibroblasts with 50 μc each of [^{3}H]glycine and [2,3-^{3}H]proline (New England Nuclear) for 24 hours. The medium was made 0.5*M* in acetic acid and dialyzed at 4°C against phosphate-buffered saline (PBS) consisting of 0.05*M* phosphate buffer, *p*H 7.1, containing 0.15*M* NaCl. Indicated amounts of antiserum were added to tubes containing 1.0 ml of ^{3}H-labeled medium (124,000 count/min per tube). Normal rabbit serum was added to give a final volume of 80 μl of serum in each tube. After incubation at 37°C for 30 minutes, an excess of goat antiserum against rabbit gamma globulin was added, and incubation was continued for 30 minutes at 37°C and then for 16 hours at 4°C. Precipitates were centrifuged at 3000*g* for 15 minutes, washed four times with PBS, and solubilized in 0.5*M* acetic acid. Absorbance measurements at 280 nm revealed that equal amounts of protein were precipitated in each tube. Portions were counted in Aquasol (New England Nuclear). For prior treatment with pepsin, medium was made 0.5*M* in acetic acid and digested for 5 hours at 15°C with pepsin (Worthington, twice crystallized) (100 μg/ml). The pepsin was inactivated by bringing the *p*H to 8.2 with NaOH, and the medium was dialyzed against PBS and titrated as above with 50,000 count/min per tube.