Assay of prolyl 4-hydroxylase by the chromatographic determination of [^{14}C]succinic acid on ion-exchange minicolumns

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An assay for prolyl 4-hydroxylase (EC 1.14.11.2) is described which measures succinic acid produced during the decarboxylation of 2-oxoglutaric acid in the presence of poly(L-Pro-Gly-L-Pro). [^{14}C]Succinic acid was separated from its precursor 2-oxo[5- ^{14}C]glutaric acid by using ion-exchange minicolumns. The contamination of succinic acid by 2-oxoglutaric acid was approx. 1%, and the recovery of succinic acid was 100%. Kinetic parameters of prolyl 4-hydroxylase measured by the assay showed good agreement with published values. Our experience indicates that the measurement of prolyl 4-hydroxylase by the production of succinic acid is especially suited to investigations involving large numbers of assays.

INTRODUCTION

Prolyl 4-hydroxylase (procollagen-proline,2-oxoglutarate 4-dioxygenase, EC 1.14.11.2) catalyses the hydroxylation of certain prolyl residues in collagen and a few other proteins with collagen-like amino acid sequences, thus permitting the formation of the characteristic triple-helical structure. The enzyme belongs to a group of dioxygenases that requires Fe^{2+} , 2-oxoglutarate, CO_2 and ascorbate in addition to the primary substrate (Kivirikko & Myllyla, 1980, 1982).

Several assay procedures have been described for prolyl 4-hydroxylase which use biosynthetic [^{14}C]proline-labelled procollagen as substrate (Peterkofsky & Udenfriend, 1965; Kivirikko & Prockop, 1967*b*; Berg & Prockop, 1973). [^{14}C]Hydroxyproline formed during the reaction is measured after acid hydrolysis of the protein, but, although specific, these procedures are time-consuming. The use of procollagen is made more convenient by labelling the protein with [^3H]proline and measuring the release of $^3\text{H}_2\text{O}$ that follows hydroxylation of the prolyl residues (Hutton *et al.*, 1966; Peterkofsky & DiBlasio, 1975). Assays using [^3H]proline-labelled procollagen are highly sensitive and can be used to measure enzyme activity in crude tissue samples. The major limitation of the procedure is the time-consuming preparation of radiolabelled procollagen. Other disadvantages are the non-uniformity of successive procollagen preparations, arising partly from the unknown extent of contamination with unlabelled hydroxylated collagen, and the relatively narrow procollagen concentration range over which the assays are linear.

A number of synthetic polypeptides can be used as substrates for prolyl 4-hydroxylase (Kivirikko & Myllyla, 1980; Prockop *et al.*, 1976). The reaction is followed by using either a chemical assay for hydroxyproline after release by hydrolysis from the peptide substrate (Kivirikko & Prockop, 1967*a*), which, although specific, has low sensitivity and is relatively tedious, or by measuring O_2 consumption (Nietfeld & Kemp, 1980). A more sensitive assay depends on measuring the release of $^{14}\text{CO}_2$ during the decarboxylation of 2-oxo[1- ^{14}C]glutarate, which is coupled to the hydroxylation reaction (Rhoads & Udenfriend, 1968). Because of other reactions involving 2-oxoglutarate in crude tissue

preparations, this assay requires the use of purified enzyme. Ensuring capture of the released $^{14}\text{CO}_2$ is somewhat tedious, and the assay is therefore relatively inconvenient for large numbers of assays.

We have developed an alternative assay for purified prolyl 4-hydroxylase which measures the formation of [^{14}C]succinate that is stoichiometrically produced from 2-oxo[5- ^{14}C]glutarate during the reaction with synthetic poly(L-Pro-Gly-L-Pro) as the primary substrate. The [^{14}C]succinate is separated from 2-oxo[5- ^{14}C]glutarate by using commercially available prepacked minicolumns, which facilitate the rapid analysis of large numbers of experimental samples.

MATERIALS AND METHODS

Materials

Prolyl 4-hydroxylase purified from the leg tissue of 17-day chick embryos by the method of Kedersha & Berg (1981) appeared homogeneous when examined by SDS/polyacrylamide-gel electrophoresis. Poly(L-Pro-Gly-L-Pro), average M_r 5200, was purchased from Miles Yeda, Rehovoth, Israel. 2-Oxo[5- ^{14}C]glutaric acid and [^{14}C]succinic acid were purchased from Amersham International, Amersham, Bucks., U.K. Strong anion-exchange minicolumns (3 ml); Bond ElutTM SAX were purchased from Analytichem International, Harbor City, CA, U.S.A. Unlabelled 2-oxoglutaric acid (disodium salt) was obtained from Boehringer, Mannheim, Germany, ferrous ammonium sulphate from BDH Chemicals, Poole, Dorset, U.K., and ascorbic acid (sodium salt), DL-dithiothreitol, catalase (crystalline suspension from bovine liver) bovine serum albumin (essentially fatty acid-free), succinic acid (disodium salt) and Tris were all from Sigma (London) Chemical Co., Poole, Dorset, U.K. [^3H]Prolyl-labelled procollagen was prepared from 17-day-embryonic chick calvaria by the method of Peterkofsky & DiBlasio (1975). The specific radioactivity of the purified procollagen was approx. 100 kBq/ng.

Enzyme assay

The reaction was carried out in a final volume of 0.5 ml, containing: 20 μM -ferrous ammonium sulphate, 2 mM-ascorbic acid, 100 μM -dithiothreitol, 50 μg of

catalase, 1 mg of bovine serum albumin, 50 μ g of poly(L-Pro-Gly-L-Pro) denatured at 100 °C for 10 min and cooled rapidly in ice immediately before addition, and 500 ng of purified prolyl 4-hydroxylase in 50 mM-Tris/HCl buffer, pH 7.6. The reaction was started by the addition of 2-oxo[5- 14 C]glutaric acid (sp. radioactivity 15 kBq/ μ mol) to a final concentration of 100 μ M, and samples were incubated at 37 °C. The reaction was stopped by the addition of 50 μ l of 70 mM- H_3PO_4 and 50 μ l of 500 mM-sodium phosphate buffer, pH 3.2, to stabilize the pH of the samples at pH 3.2. Bond Elut™ strong anion-exchange minicolumns were prewashed according to the manufacturer's instructions with 2 ml of methanol, followed by 2 ml of distilled water and 2 ml of 50 mM-sodium phosphate buffer, pH 3.2. A sample (0.5 ml) was applied to the column and eluted with 2.5 ml of 50 mM-sodium phosphate buffer by using a reduced pressure of 30 kPa. Fractions were added to 10 ml of Beckman CP high-capacity scintillation fluid and counted for radioactivity. The simultaneous elution of 20 minicolumns was made possible by the construction of a modified version of the Analytichem Vac Elut™ system, adapted to hold the 10 ml scintillation vials used to collect and count the column fractions for radioactivity.

The columns were regenerated by washing with 10 ml of 50 mM-sodium phosphate buffer, pH 3.2, containing 1 M-NaCl, followed by 10 ml of 50 mM-sodium phosphate buffer, pH 3.2, and were stored in 50 mM-sodium phosphate buffer, pH 3.2, containing 0.2% benzalkonium chloride as a preservative at 4 °C. Before re-use, the columns were washed with a further 5 ml of 50 mM-sodium phosphate buffer, pH 3.2.

Assay of enzyme activity by measuring the release of $^3\text{H}_2\text{O}$ from [^3H]proline-labelled procollagen was carried out by the method of Peterkofsky & DiBlasio (1975).

RESULTS AND DISCUSSION

The decarboxylation of 2-oxoglutarate is coupled stoichiometrically to the hydroxylation of procollagen and its synthetic analogues in the prolyl 4-hydroxylase reaction, and the measurement of the product succinate therefore affords a direct estimate of enzyme activity. We have used ion-exchange chromatography to separate succinic acid from 2-oxoglutaric acid, on the basis of the difference in the pK_a values of the two acids.

Initial studies to establish the optimum conditions for the separation of the two acids on ion-exchange minicolumns were carried out by adding to the assay mixture known concentrations of 2-oxo[14 C]glutaric acid and [14 C]succinic acid. Maximum resolution of the two acids was obtained by eluting the columns with 50 mM-sodium phosphate buffer, pH 3.2 (Fig. 1). At this pH 2-oxoglutaric acid (pK_a values 2.5 and 5.3, estimated from the pK_a values for pentanedioic acid and 2-oxobutanoic acid; Fasman, 1976) exists predominantly as the monocarboxylate anion and binds preferentially to the cationic sites of the anion-exchange resin, whereas succinic acid (pK_a values 4.2 and 5.6; Fasman, 1976) remains essentially neutral and is not retained. The separation of the acids was highly dependent on pH, and it is therefore important to check the pH of all buffers before each experiment. The recovery of succinic acid from the columns was directly proportional to the amounts loaded on the columns. Of the total succinic acid loaded on the column, 98% was recovered in the first

Fig. 1. Separation of [14 C]succinic acid and 2-oxo[14 C]glutaric acid on anion-exchange minicolumns

The assay mixture was spiked with 100 μ M-[1- 14 C]succinic acid and 100 μ M-2-oxo[5- 14 C]glutaric acid (sp. radioactivities 15 kBq/ μ mol) and separated on the anion-exchange minicolumns as described in the Materials and methods section. Succinic acid was eluted in the narrow, first, peak and 2-oxoglutaric acid in the broad, second, peak.

Fig. 2. Dependence of initial rate of succinic acid production on concentration of prolyl 4-hydroxylase

The rate of production of [14 C]succinate in the enzymic reaction was directly proportional to the concentration of enzyme.

3 ml of eluate; the remaining 2% appeared in the following 2 ml of eluate. Approx. 1% of the 2-oxoglutaric acid sample was eluted from the column with the succinic acid. The contaminating material was found to migrate separately from the main 2-oxoglutaric acid peak, and probably represented a neutral impurity in the 2-oxoglutaric acid sample. The low contamination had negligible effect on the accuracy of the succinic acid measurement.

The assay procedure was validated by some basic measurements on the kinetic properties of prolyl 4-hydroxylase. Four separate determinations of the K_m for 2-oxoglutarate gave a mean value ($\pm$ S.E.M.) of 21.4 ± 1.6 μ M. The K_m for 2-oxoglutarate measured by the [^3H]proline-labelled-procollagen-based method of

Fig. 3. Rate of production of [^{14}C]succinic acid with time

Under the standard reaction conditions defined in the Materials and methods section, the production of [^{14}C]succinate in the prolyl 4-hydroxylase reaction was linear with time over 30 min.

Peterkofsky & DiBlasio (1975) was $21.75 \pm 1.9 \mu\text{M}$. These values compare with a published value of $22 \mu\text{M}$ based on a method using poly(L-Pro-Gly-L-Pro) (Myllyla *et al.*, 1977). Our measurement of the K_m of Fe^{2+} was $2 \mu\text{M}$, which also showed good agreement with published values (Tuderman *et al.*, 1977). The rate of reaction determined by measuring the formation of [^{14}C]succinate was directly proportional to the enzyme concentration (Fig. 2).

The dependence of the reaction rate on the concentration of poly(L-Pro-Gly-L-Pro) was determined by using a hyperbolic curve-fitting programme (Cunliffe & Franklin, 1986) and was found to obey Michaelis-Menten kinetics at low substrate concentrations. Substrate inhibition was observed at high substrate concentrations, a property which has also been reported for other synthetic peptide substrates (Myllyla *et al.*, 1977). Under the standard reaction conditions outlined in the Materials and methods section, the reaction was linear with time during a 30 min incubation period (Fig. 3).

The mean concentration ($\pm \text{S.E.M.}$) of succinate produced in ten replicate incubations (20 min) in a single experiment was $3.17 \pm 0.10 \text{ nmol}$; that produced in duplicate incubations (20 min) in eight experiments was $3.39 \pm 0.23 \text{ nmol}$.

The ion-exchange minicolumns could be regenerated for further use by washing with high-ionic-strength buffer. Repeated use was found to result in a gradual loss of resolution of the two peaks; however, the columns

could be reused at least twice without any appreciable deterioration of the separation profile.

The results indicate that the measurement of succinic acid is a valid means of assaying prolyl 4-hydroxylase activity. The use of commercially available reagents and the rapid separation of succinic acid using preppacked minicolumns afford a simple and convenient alternative to existing procedures. The new assay is particularly suitable when rapid analysis of large numbers of samples is an important consideration. We have used the assay in several thousand tests of potential inhibitors of prolyl 4-hydroxylase and have found it to be robust and reliable.

We thank Dr. Ross Stein, ICI Americas Inc., for a suggestion that led us to initiate the work described in this report, and Dr. Geoffrey Tomalin, Department of Biochemistry, University of Manchester, for his advice and guidance.

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