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Separation of Coenzyme A and Its Precursors by Reversed-Phase High-Performance Liquid Chromatography

OLA HALVORSEN AND SVERRE SKREDE

Institute of Clinical Biochemistry, Rikshospitalet, University of Oslo, Oslo, Norway

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Dephospho-CoA and CoA can be completely separated by reversed-phase high-performance liquid chromatography using an isocratic elution mode. The quantitation of picomole amounts of dephospho-CoA is possible in this system, using CoA as an internal standard. The other precursors of CoA, i.e., pantothenic acid, 4′-phosphopantothenic acid, 4′-phosphopantothenoyl-1-cysteine, and 4′-phosphopantetheine are also completely separated by reversed-phase high-performance liquid chromatography using straight elution modes. Separation by high-performance liquid chromatography in one run of all the precursors in the biosynthesis of CoA and of CoASH, CoASS, and dephospho-CoASS can be accomplished by a methanol gradient.

In mammals, the biosynthesis of CoA proceeds through five enzymatic steps, starting with pantothenic acid (1). The first reaction is phosphorylation of pantothenic acid to 4′-phosphopantothenic acid. Then a condensation with 1-cysteine occurs, giving 4′-phosphopantothenoyl-1-cysteine, which is subsequently decarboxylated to 4′-phosphopantetheine. ATP is then utilized for condensation of 5′-AMP with 4′-phosphopantetheine to form dephospho-CoA, which finally is phosphorylated at the 3′ position of the ribose moiety giving CoA.

Paper chromatography (2,3), separation with ion-exchange chromatography (3), and methods more or less specific for CoA (4–6) have been used in studies on the metabolism of CoA and its precursors. All chromatographic systems used, however, separate these intermediates only partially. Thus CoA and dephospho-CoA cannot be efficiently separated, and also 4′-phosphopantothenic acid, 4′-phosphopantothenoyl-1-cysteine, and 4′-phosphopantetheine overlap in these systems (7).

We now report methods utilizing reversed-phase high-performance liquid chromatography which separate CoA completely from dephospho-CoA and also separate 4′-phosphopantothenic acid, 4′-phosphopantothenoyl-1-cysteine, and 4′-phosphopantetheine efficiently from each other.

MATERIALS AND METHODS

Reagents

CoA (of about 85% purity) was obtained from Boehringer–Mannheim GmbH, Mannheim, Federal Republic of Germany, and Chomatopure CoA and dephospho-CoA were obtained from P-L Biochemicals, Milwaukee, Wisconsin. [G-^{14}C]Phosphopantothenoyl-1-cysteine was a generous gift from professor Roberto Scandurra, Universita di Roma, Cattedra di Biologia Molecolare Della Facolta di Medicina, Citta Universitaria, Rome, Italy. [G-^{3}H]-CoA and (+)-[1-^{14}C]pantothenic acid were provided by New England Nuclear (Boston, Mass.). Nucleotide pyrophosphatase (EC 3.6.1.15) from *Crotalus atrox* venom, type 3, was purchased from Sigma Chemical Company (St. Louis, Mo.). Thio-

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propyl-Sepharose 6B was a product of Pharmacia Fine Chemicals AB (Uppsala, Sweden). Other chemicals were commercial products of high purity.

Equipment

A DuPont 830 high-performance liquid chromatograph with a DuPont 837 spectrophotometer was used. A Reodyne 7120 syringe-loading sample injector (50 μl) allowed injection at atmospheric pressure. The column, 30 cm long, 3.9-mm i.d., was a μBondapak C_{18} with a 10-μm particle size, provided by Waters Associates, Massachusetts. The concentration of methanol in the hplc[1] gradient elutions was checked by gas chromatography with ethanol as internal standard, using a Varian chromatograph Model 2100 (Varian Aerograph Walnut Creek, Calif.) equipped with a U column of glass (6 ft, ¼-in. i.d.) and a hydrogen flame-ionization detector. The carrier gas was nitrogen (30 ml/min). The injection temperature was 254°C, and the detector was kept at 250°C. The column material used was Porapak Q (mesh 80–100) (Waters Associates, Mass.).

Mobile Phase Preparation

Phosphate buffers (fc 20 mmol/liter) were prepared from potassium dihydrogenphosphate and adjusted to the desired pH with KOH (2 mol/liter) or concentrated phosphoric acid. The buffers and methanol were degassed separately for 5 min at lowered pressure. Filtering was performed with a Millipore filter type HA (0.45 μm) for the aqueous solution and FH (0.5 μm) for methanol. Methanol was then added to the aqueous solution until the desired concentration was obtained. The following mobile phase solvents were prepared: (A) Phosphate buffer (pH 5.0):methanol (90:10), (B) phosphate buffer (pH 5.0):methanol (70:30); (C) phosphate buffer (pH 3.5); (D) phosphate buffer (pH 4.0); (E) phosphate buffer (pH 8.25):methanol (55:45).

[1] Abbreviation used: hplc, high-performance liquid chromatography.

Sample Preparation

All samples, i.e., standards prepared as described below, were centrifuged at 27,000*g* for 10 min. The supernatants were then taken to dryness and dissolved in the respective mobile phase. All commercial standards were dissolved directly in the mobile phase. If necessary, the pH of the sample was adjusted to that of the mobile phase with HCl (0.1 mol/liter) or KOH (0.1 mol/liter).

Preparation of Isotopically Labeled Standards

4′-Phosphopantothenic acid. Dialyzed rat liver supernatant (1 ml), prepared in phosphate buffer (20 mmol/liter, pH 7.2) as described previously (8), was incubated for 2 h with (+)-[1-^{14}C]pantothenic acid (0.2 mmol/liter sp act 20 nCi/nmol), ATP (10 mmol/liter), $MgCl_2$ (5 mmol/liter), dithiothreitol (2 mmol/liter), and Tris buffer (50 mmol/liter pH 7.4) in a total volume of 2.5 ml. The supernatants after precipitation of protein with methanol were subjected to chromatography on Whatman paper No. 3, in butan-1-ol/acetic acid/water (5/2/3) (2). Elution of 4′-phosphopantothenate from the paper (R_f 0.5) was performed with water.

4′-Phosphopantetheine. [G-^{3}H]CoA (2 mmol/liter, sp act 10 nCi/nmol) was incubated in Tris buffer (50 mmol/liter, pH 7.0) with nucleotide pyrophosphatase from *Crotalus atrox* venom, type 3 (8 units), $MgCl_2$ (2 mmol/liter), and dithiothreitol (0.1 mmol/liter), in a final volume of 1 ml, for 30 min at 37°C. The reaction medium was cooled on ice and subjected to chromatography on thiopropyl-Sepharose 6B at 4°C. The procedure was as follows: 3 ml of gel material was equilibrated with potassium phosphate buffer (20 mmol/liter, pH 8.0). The cooled incubation medium was

diluted to 2 ml with the equilibrium buffer, and the pH was adjusted to 8.0 with 0.1 N NaOH. The thiols of the medium were then allowed to react for 30 min by a thiol–disulfide exchange reaction with the thiopropyl-protected disulfide groups (35 mmol/liter). The column was washed with 10 ml of potassium phosphate buffer (20 mmol/liter, pH 7.0). Elution was performed with dithiothreitol (0.1 mol/liter) in a potassium phosphate buffer (20 mmol/liter, pH 8.0).

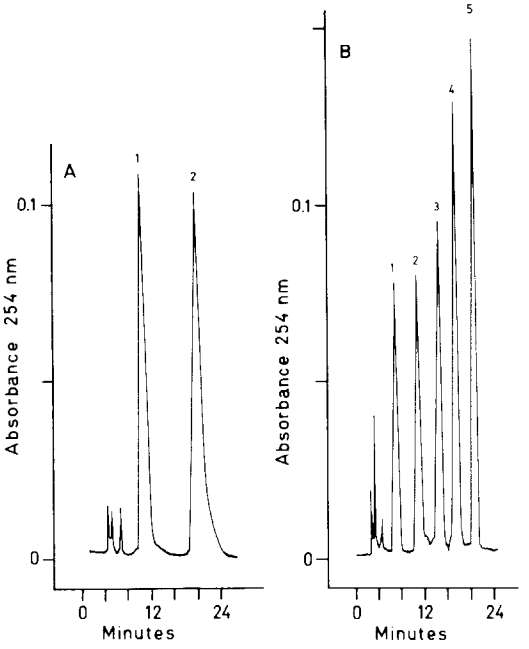

FIG. 1. (A) Separation of CoA (1) and dephospho-CoA (2). The substances (11–17 nmol) were injected in a volume of 50 μl into mobile phase A (potassium phosphate buffer (20 mmol/liter, pH 5.0):methanol (90:10)). The flow rate was 0.93 ml/min, and the temperature was 22°C. The retention times were 9 min for (1), and 18.5 min. for (2). The uv absorption was monitored at 254 nm. Full-scale deflection represented an absorbance of 0.16. (B) Gradient separation of CoASH (1), dephospho-CoASH (2), CoASS (3), dephospho-CoASS (5), and the mixed disulfide of CoA and dephospho-CoA (4). The substances (6–13 nmol) were injected in a volume of 50 μl into mobile phase A (see A). Then, a methanol gradient from 10 to 20% was developed during 25 min by admixture of solvent B (potassium phosphate buffer (20 mmol/liter, pH 5.0):methanol (70:30)). (The concentration of methanol in the gradient was checked with gas–liquid chromatography.) The flow rate was 1.1 ml/min and the temperature was 22°C. The retention times were 6.7, 10.5, 14.3, 17.2, and 20.4 min for (1)–(5), respectively. Scale as in (A).

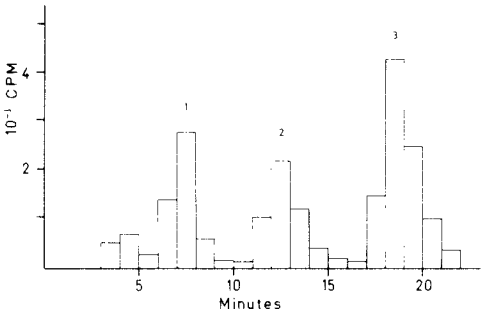

FIG. 2. Isocratic separation of 4′-phosphopantothenic acid (1), 4′-phosphopantothenoyl-1-cysteine (2), and 4′-phosphopantetheine (3). Isotopically labeled standards prepared as described under Materials and Methods were injected in a volume of 50 μl and chromatographed in mobile phase C (potassium phosphate buffer (20 mmol/liter, pH 3.5)) with a flow rate of 0.95 ml/min. The temperature was 22°C. Fractions of 0.95 ml were collected from the column effluent and assayed by liquid scintillation.

4′-[G-^{14}C]Phosphopantothenoyl-1-cysteine. The barium salt (9) was dissolved in potassium phosphate buffer (20 mmol/liter, pH 3.5) and $BaSO_4$ was precipitated with an equal amount of Na_2SO_4 (15.9 mmol/liter). The precipitating solution also contained dithiothreitol 10 mmol/liter, and after precipitation, the sodium salt was filtered through a Millipore Swinnex 13(R) filter with 0.45-μm pore size.

Preparation of the disulfides and the mixed disulfide of CoA and dephospho-CoA. CoA or dephospho-CoA separately, or equal amounts of each were dissolved in a concentration of 0.26 mmol/liter in potassium phosphate buffer (20 mmol/liter, pH 8.0). Oxidation of the thiols to the corresponding disulfides was carried out by exposure to atmospheric oxygen at 37°C for 5 h.

RESULTS AND DISCUSSION

The separation of CoA and dephospho-CoA under isocratic conditions is shown

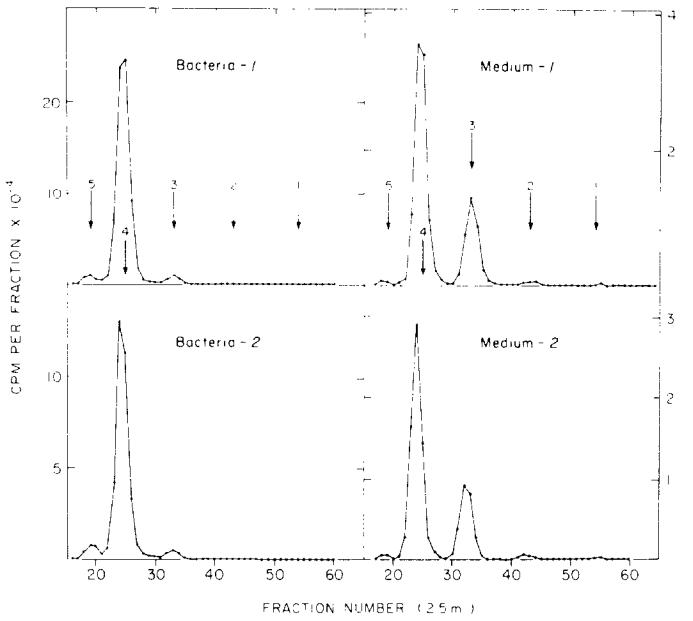

FIG. 1. Bio-Gel P4 elution profiles of azo-dyes of labeled pABAglu$_n$ derived from *Corynebacterium* folates. *Corynebacterium* was cultured in the presence of [^{14}C]pABA and labeled folates in the bacteria and medium were converted to pABAglu$_n$ by the complete cleavage procedure (*1*) and by Zn/HCl treatment (*2*) as described under Materials and Methods. The resulting pABAglu$_n$ was converted to azo-dye derivatives, which were purified by chromatography on Bio-Gel P2, and were separated, according to glutamate chain length, by chromatography on Bio-Gel P4, as described under Materials and Methods. The numbers *1*–*5* indicate the elution positions of azo-dyes of pABAglu$_1$ – chromatographed under identical conditions.

folate cleavage to unsubstituted pABAglu$_n$ was assessed by conversion of the products to azo-dye derivatives and the binding of the azo-dyes to Bio-Gel P2 at acid pH and their elution at neutral pH (4).

The complete procedure increased the yield of pABAglu$_n$ derived from labeled intracellular folates from 60 to 90% and that from extracellular folates from 77 to 84%. The recoveries may be underestimated as *Corynebacterium* may also metabolize pABA to nonfolate compounds. In a similar study on the metabolism of [^{3}H]PteGlu by *Lactobacillus casei* (Lebowitz and Shane, in preparation), 97.1 ± 3.5(4)% of the labeled bacterial folates were recovered as unsubstituted pABAglu$_n$ following the complete cleavage procedure. Ion-exchange chromatography of the cleavage products demonstrated that the only labeled products were pABAglu$_n$ and a pteridine derivative.

The Bio-Gel P4 elution profiles of azo-dyes of pABAglu$_n$ obtained after cleavage of *Corynebacterium* folates by the two methods are shown in Fig. 1. The profiles were qualitatively indistinguishable.

DISCUSSION

Cleavage of folate derivatives at the C,9–N,10 bond greatly simplifies the subsequent analysis of the glutamate chain lengths of the derivatives by ion-exchange or gel chromatography (1–4). However, the usefulness of this technique is dependent on the quantitative cleavage of folate derivatives to unsubstituted pABAglu$_n$, a homologous series of compounds differing only in glutamate chain length. Incomplete cleavage, or cleavage to more than one family of compounds would complicate the subsequent identification of glutamate chain

15 min before a combined methanol/pH gradient was started by admixture of solvent E. The methanol gradient was linear from 0 to 20% methanol in 35 min, and the pH gradient was sigmoid-shaped with a change in pH from 4.0 to 6.5 during 36 min. This pH gradient was necessary to obtain satisfactory separation and bandwidth for CoASH. With the pH at about 3.5–4.0 in the solvent, CoA was incompletely separated from dephospho-CoA.

A satisfactory method for the quantitative estimation of dephospho-CoA has not been described previously, but a hplc method for the quantitation of CoA was recently published (10). We have estimated dephospho-CoA using CoA as an internal standard with mobile phase A. The standard curve for different amounts in the picomole to nanomole range of dephospho-CoA is shown by Fig. 4.

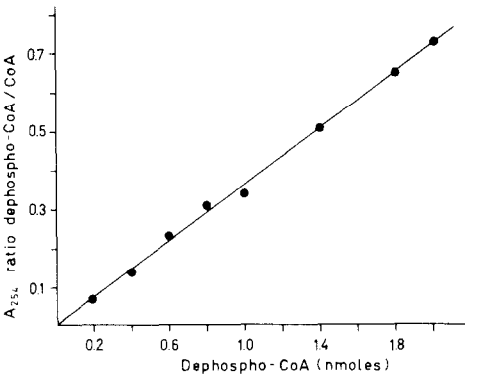

FIG. 4. Standard curve for the quantitation of dephospho-CoA with CoA as an internal standard. Different amounts of dephospho-CoA from 200 pmol to 2 nmol, together with 1.58 nmol of CoA, were injected in a volume of 50 μl into mobile phase A (cf. Fig. 1A). The operating conditions of the chromatograph were as in Fig. 1A. Abscissa: Amounts (nmol) of commercial dephospho-CoA as estimated by weight. Ordinate: Ratio of absorbances of dephospho-CoA/CoA at 254 nm calculated from peak heights.

The mechanisms responsible for solute retention in reversed-phase chromatography are incompletely understood (11). With all the mobile phases used here as primary solvents, CoASH elutes before dephospho-CoASH, the only difference between the molecules being the ionizable ribose 3′-phosphate group in CoA. A dominating difference between the thiols and their corresponding disulfides of CoA and dephospho-CoA is the double size of the disulfides, which are always eluted after the thiols.

The effects by the number of ionizable groups and molecular size on the retention time—as observed in the present study—might be predicted by the theory for binding to hydrophobic columns set forward by Horvath et al. (12).

The technique of ion suppression (13) was used to separate the compounds in Fig. 2, i.e., 4′-phosphopantothenic acid, 4′-phosphopantothenoyl-l-cysteine, and 4′-phosphopantetheine. There is a lower limit at pH 3.5 for optimal separation, however. A further decrease of pH will cause confluence of 4′-phosphopantothenoyl-l-cysteine and 4′-phosphopantetheine, with a complete lack of separation at a pH of about 2.5. The reason for this may be that the ionization of the COOH group of the l-cysteine moiety of 4′-phosphopantothenoyl-l-cysteine has been repressed at this pH. No reports have appeared on determining the pK_a values of the different acidic groups of this intermediate. In fact, hplc with nonpolar stationary phases may be used for measurements of pK values of different functional groups in a molecule (13).

Methods for separation of all the precursors of CoA as introduced by the present study may open possibilities for more detailed investigations on the mechanisms regulating the biosynthesis of CoA. The regulatory mechanisms in the cytosolic pathway (1) as well as in the mitochondrial system described quite recently (8) are only incompletely understood.

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