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Dephospho-CoA Kinase Provides a Rapid and Sensitive Radiochemical Assay for Coenzyme A and Its Thioesters

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Abstract

A new approach to determine *in vivo* pools of coenzyme A and short chain acyl-CoA thioesters is reported. The metabolites released by extraction with trichloroacetic acid are recovered and quantitatively dephosphorylated by treatment with shrimp alkaline phosphatase. Following phosphatase removal, the dephosphorylated CoA metabolites are quantitatively rephosphorylated by treatment with γ -labeled ³³P-ATP plus a dephospho-CoA kinase. The resulting radioactive CoA metabolites are then separated by reverse-phase high-performance liquid chromatography and quantitated by scintillation counting. Due to the enzymatic radio-phosphorylation, the assay is specific for CoA and its short chain thioesters and sensitive to subpicomole levels of these compounds.

Introduction

Many compounds produced by metabolic engineering of microbial cells are derived from thioesters of coenzyme A (CoA), such as acetyl-CoA, malonyl-CoA and succinyl-CoA. Therefore, the pool sizes and metabolic fluxes of CoA and its thioesters are important factors that must be monitored for efficient production of such compounds as polyketides and other antibiotics. The methods currently available for analysis of intracellular short-chain CoA esters are problematical for various reasons. Biosynthetic labeling by feeding a radioactively labeled CoA precursor to cell cultures followed by measurement of the levels of radioactivity in CoA and its esters [1;2;3;4] provides a specific and highly sensitive approach. However, this method is generally restricted to organisms that are auxotrophic for such precursors and able to grow in chemically-defined culture media. Moreover, even given a suitable organism and growth medium, such *in vivo* labeling methods cannot be used to monitor production of CoA esters in facilities such as fermentors due to radioactive contamination of the equipment and the need to properly dispose of the large volumes of radioactive medium that would be generated. Chemical analysis of CoA and its esters by HPLC with detection by UV absorption of the CoA adenine moiety [5] although generally applicable, is insensitive. Moreover, analysis and quantitation are complicated by the presence of numerous other intracellular metabolites that absorb at the same wavelengths. Greater sensitivity and somewhat improved specificity is provided by derivatization of CoA and other adenine-containing compounds to their fluorescent 1, N6-ethenoadenine derivatives [6;7], but this approach requires specialized

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equipment for on-column derivatization and use of carcinogenic compounds, some of which must be synthesized. Other methods require expensive dedicated instruments such as mass spectrometers and lack demonstrated applicability to a range of intracellular CoA esters [8]. We report a new method based on the use of dephospho-CoA kinase (DPCK), the enzyme that catalyzes the last step of CoA biosynthesis [9], the ATP-dependent phosphorylation of the 3'-hydroxyl group of the ribose moiety of dephospho-CoA. In our approach, the intracellular CoA esters are extracted and then freed of the extraction reagent. The extracted compounds are then treated with a non-specific phosphatase (phosphomonoesterase) to release the 3'-phosphates of CoA and its esters as inorganic phosphate. After removal of the phosphatase, the 3'-phosphate is replaced with ^{33}P by treatment with DPCK and $[\gamma\text{-}^{33}\text{P}]\text{ATP}$ (Fig. 1). The resulting ^{33}P -labeled CoA compounds are then separated by reverse-phase HPLC and quantitated by scintillation counting. The specificity of DPCK restricts the radioactive labeling to CoA and its thioesters.

Materials and Methods

Chemicals, enzymes, media, and bacterial strains

CoA, dephospho-CoA, succinyl-CoA, acetyl-CoA, malonyl-CoA, n-heptadecanoyl CoA, phosphoenolpyruvate (PEP), NADH, pyruvate kinase (PK)/lactate dehydrogenase (LDH) enzymes from rabbit muscle, and ATP were purchased from Sigma Chemical Co. (St. Louis, MO). Artic shrimp alkaline phosphatase (SAP; specific activity 2200 U/mg) was purchased from Roche, Inc. (Indianapolis, IN) and Antarctic shrimp phosphatase was purchased from New England Biolabs (Beverly, MA). American Radiolabeled Chemicals, Inc. (St. Louis, MO) supplied $[\gamma\text{-}^{33}\text{P}]\text{ATP}$ (specific activity: 3000 Ci/ mmol) and $\beta\text{-}[3\text{-}^3\text{H}]\text{alanine}$ (specific activity: 50 Ci/ mmol). Vivaspin 500 columns were purchased from ISC Bioexpress (Kaysville, UT). Bond Elut Jr. C18 columns were purchased from Varian (Walnut Creek, CA). The $\mu\text{Bondapak}^{\text{TM}}$ C18 HPLC column (125 Å pore size, 10 µm particle size, 4.6 × 250 mM cartridge) was purchased from Waters Corporation (Milford, MA). Slide-A-Lyzer dialysis cassettes and 5 mL protein purification columns were purchased from Pierce Biotechnology, Inc. (Rockford, IL). The Ni-NTA agarose was purchased from Qiagen (Valencia, CA). All other chemicals were purchased from Sigma.

Strain SI92, a *ApanD* derivative of *Escherichia coli* K-12 strain lacking aspartate-1-decarboxylase was provided by Dr. S. Iram [10]. The *E. coli* and *Aquifex aeolicus* DPCK genes (DPCK_E and DPCK_A, respectively) were cloned into vector pET28b and expressed in strain *E. coli* BL21(DE3). The following primers used for the PCR amplification of DPCK_A were obtained from Integrated DNA Technologies (IDT, Coralville, IA): Forward: 5'-GGGAATTCCATATGGGACATAACCGCAGGGCTTGTAATA-3' Reverse: 5'-CGCGGATCCAAGCTTTCAAGGGTCTCTTGAGTTCTTCGTAA-3' The primers for amplification of DPCK_E were: Forward: 5'-GGGAATTCCATATGAGGTATATAGTTGCCTTAACGGGAG-3' Reverse: 5'-CGCGGATCCAAGCTTTTACGGTTTTTCTGTGAGACAAACTGC-3' *A. aeolicus* genomic DNA was obtained from the American Type Culture Collection. Purified human phosphopantetheine adenylyltransferase/dephospho-CoA kinase was the kind gift of Dr. A. Osterman of the Burham Institute, La Jolla, CA [11]. A unit of DPCK is 1 µmole of product formed per min.

Minimal E medium contained magnesium sulfate heptahydrate (8 gm/L), citric acid monohydrate (80 gm/L), potassium phosphate dibasic anhydrous (400 gm/L), and sodium ammonium phosphate tetrahydrate (140 gm/L).

Dephosphorylation and Phosphorylation Reactions

All commercial CoA and CoA esters were used at a concentration of 1 mg/mL. Dephosphorylation of CoA and CoA-esters was performed by incubating 500 µg of the compound in SAP buffer (50 mM Tris-HCl, 5 mM MgCl₂, pH 8.5) containing 5 units of shrimp alkaline phosphatase (SAP) in a total volume of 555 µl. Following the completion of the reaction, SAP was removed from the sample by ultrafiltration using a Vivaspin 500 (5,000 MW cut off) cartridge at the maximum recommended speed (15,000 x g) for 10 min.

The Vivaspin cartridge-purified and dephosphorylated CoA and CoA esters were rephosphorylated using approx. 9 units of human DPCK/PPAT (DPCK-*Hs*) for spectrophotometric assay by ADP production or ~3 µg *E. coli* DPCK (DPCK-*Ec*) for assay by HPLC. The DPCK reaction buffer was composed of 4 µM ATP, 50 mM Tris-HCl (pH 7.5), 2 mM MgCl₂ and 20 mM KCl (pH 7.0) in a final volume of 500 µl.

Assay of the phosphorylation reaction by spectrophotometry

The phosphorylation reaction was followed by spectrophotometry using a modification of the standard enzyme-coupled method of Daugherty et al [11;12]. Briefly, 100 µl of a dephosphorylated sample was incubated with 2 mM phosphoenolpyruvate, 0.3 mM NADH, and 4 µM ATP in the rephosphorylation reaction buffer. The reaction was monitored spectrophotometrically (Beckman DU-640) at room temperature as a decline in absorbance at 340 nm. DPCK-*Hs* (~ 9 units) and 5 µl pyruvate kinase/lactate dehydrogenase (~ 6.5 units and 3 units, respectively) were added after the sample had been running for forty and eighty seconds, respectively.

Analysis by high performance liquid chromatography (HPLC)

To confirm the enzyme-coupled assay a dephosphorylated and phosphorylated CoA and CoA esters produced by the reactions above were separated via high performance liquid chromatography (HPLC) after 10 min of room temperature incubation with DPCK-*Ec*. Separation of products was performed on a Waters µBondapak C18 HPLC column at room temperature and a flow rate of 1 mL per minute. The conditions for the separation were modified from Roughan [13]. The starting conditions were 100% 50 mM ammonium acetate (to pH 5.0 with glacial acetic acid), followed by a gradient of 30% acetonitrile and 70% ammonium acetate over 40 min, and then 100% acetonitrile over 5 min. A radioactive phosphorylation reaction was also performed using 1.5 nmol γ-³³P-ATP (300 Ci/mmol) as the substrate. The radioactive products were detected using a Beckman Coulter LS 6500 scintillation counter. The same method was used to analyze the composition of CoA metabolites in *E. coli* extracts.

Extraction of intracellular CoA metabolites

The CoA pools of *E. coli* cultures were extracted with trichloroacetic acid using essentially the method of Roughan [13]. We chose the Roughan procedure since it was specifically designed to preserve malonyl-CoA, the most labile of the CoA thioesters found in *E. coli* [13]. Extraction with chaotrophic acids has long been known to completely release the intracellular contents of *E. coli* cells [7;14;15;16]. The protocol for biosynthetic labeling was that of Iram and Cronan [10] *E. coli* strain SI92 was grown on minimal E plates supplemented with 20 µg/ml chloramphenicol, 0.01% methionine, 0.2% glucose, and 0.5 µM β-alanine overnight at 37°C. The cells were then plated on minimal E plates supplemented as above, but lacking β-alanine to deplete the preexisting CoA pools. A liquid culture was then inoculated from the starved cells from the plates lacking β-alanine into the medium above that contained 0.5 µM ³H-β-alanine (50 Ci/mmol) and the cultures were grown overnight at 37°C. After 16 h of growth, 1 mL of culture was combined with 60 µl of 100% solution of trichloroacetic acid in a 2 mL microcentrifuge tube and the solution was mixed by inversion. The tube was put on ice for 3

min and then centrifuged at 4°C for 3 min. The deproteinized supernatant was transferred to a fresh microcentrifuge tube and the pellet was again extracted with 1 ml of 1% trichloroacetic acid [13]. The supernatants from both extractions were combined and loaded onto a C18 cartridge that had been equilibrated first with 2 ml of 100% methanol and then with 8 ml of 1 mM HCl. The cartridge was washed with 6 ml of 1 mM HCl to remove trichloroacetic acid and the CoA and CoA esters were then eluted with 3 ml 0.1 M ammonium acetate in 65% ethanol [13]. This eluate was distributed (~500 µl each) among six 2 ml microcentrifuge tubes and these samples were evaporated under vacuum at room temperature until the total volume remaining was less than 300 µl. Ammonium acetate (50 mM, pH 5.0) was then added to give a final volume of 500 µl [13;17].

CoA Labeling Reactions

To test the ability of the DPCK CoA to assay the CoA pools of *E. coli*, 500 µl of each cellular extract was dephosphorylated as per the dephosphorylation reaction above. That sample was then phosphorylated with radiolabeled ATP by DPCK-*Ec*. The phosphorylated sample was then separated via HPLC as above and run four times; each run contained an internal standard of with 50 µg of CoA or of a CoA thioester. The internal standard peak from each run was collected and the individual ³H and ³³P radioactive emissions were assayed using a Beckman Coulter LS 6500 Multi-Purpose Scintillation Counter.

Production of DPCK

DPCK-*Ec* and DPCK-*Aa* were cloned from their respective genomic DNAs via PCR amplification and the products were ligated into the NdeI and HindIII sites of pET28b. The His-tagged proteins were then obtained by the protocol of Nazi et al. [18] as developed for the *E. coli* enzyme. The activity of DPCK-*Aa* was 145.5 units per mg protein and the identities of the products were verified by HPLC demonstration of phosphorylation of dephosphorylated CoA compounds isolated from *E. coli*. The rephosphorylation reactions were run as above with 2 µg DPCK-*Aa* and incubated for 1 min at 55°C.

Results and Discussion

DPCK is active on short chain thioesters of CoA

Development of the reported assay required study of the specificity of DPCK for dephospho-CoA thioesters. Although several crystal structures of DPCKs from diverse sources have been reported [19;20], no structure of a complex of dephospho-CoA with a DPCK is as yet available. However, homology-based modeling of the DPCK-dephospho-CoA complex based on the complexes with ADP and ATP [20] predicted that the dephospho-CoA thiol would be far removed from the DPCK active site. Hence, we expected that DPCK should be active on substrates in which the dephospho-CoA thiol carried an acyl group. We therefore used phosphatase treatment to prepare the dephosphorylated derivatives of commercial samples of acetyl-CoA, malonyl-CoA, and succinyl-CoA and assayed the activity of human DPCK ((DPCK-*Hs*) on these substrates by monitoring ADP production with a standard enzyme-coupled spectrophotometric assay. All of the thioester substrates were as rapidly and completely phosphorylated as was dephospho-CoA (Fig. 2). Although the data of Fig. 2 were obtained with the bifunctional human DPCK/phosphopantetheine adenylyltransferase enzyme, similar data were obtained with the DPCKs from *E. coli* (DPCK-*Ec*) and *A. aeolicus* (DPCK-*Aa*) (data not shown). In all cases there was no significant NADH oxidation when one of the components was omitted from the reaction (data not shown).

Preliminary data using the dephospho derivative of n-heptadecanoyl CoA (C17 CoA) as a substrate showed similar results with DPCK-*Aa*. However, DPCK-*Ec* was not active on this substrate. However, efficient dephosphorylation of the long chain acyl-CoAs was very erratic

despite use of several different phosphatases. We expect that this is due to inactivation of the phosphatases by the very potent detergent properties of long chain acyl-CoAs [21;22]. The activity of DPCK-Aa on the dephosphorylation product can be explained by the fact that long chain dephospho-CoA molecules will be much less amphipathic and hence weaker detergents than the parental molecules [23], although apparently sufficiently strong to inactivate DPCK-*Ec*. Since our primary interest was in the short chain CoA species, we did not further pursue analysis of long chain species. However, if a resistant phosphatase or conditions to protect sensitive phosphatases from inactivation can be found the DPCK method could be extended to these compounds.

The DPCK and phosphatase reactions with short chain acyl-CoAs were also monitored by reverse-phase HPLC (Fig. 3). The commercial CoA sample eluted from the reverse-phase column as a single peak with a retention time of about 16 min whereas the dephospho-CoA peak generated by phosphatase treatment (as well as commercial dephospho-CoA) eluted from the column a few minutes later (retention time of about 18 min) as expected from the greater hydrophobicity resulting from loss of the 3'-phosphate. Treatment of dephospho-CoA with DPCK quantitatively shifted the peak to the same elution time as the original CoA sample. Similar results were obtained for each of the CoA thioesters, although the exact retention times varied slightly (Fig. 3). In each case, the 3'-phosphate was quantitatively removed by phosphatase treatment and quantitatively restored by DPCK treatment. It should be noted that we have used several different alkaline phosphatases with equivalent results. Although we originally used the Arctic shrimp enzyme because it was readily inactivated by heating, this property was not of advantage because even the mild heat treatment needed to inactivate the enzyme resulted in some loss of malonyl-CoA, the most labile of the thioesters [7;13]. Although the stability of CoA thioesters to alkaline conditions is markedly decreased in the presence of magnesium ion [24], a component of the Arctic shrimp phosphatase buffer, an enzyme having a lower pH optimum seemed a better choice. After we had established our assay a new phosphatase from Antarctic shrimp became commercially available. Although this enzyme has a pH optimum of 6.0 and is more readily heat-inactivated than the Arctic shrimp alkaline phosphatase, our preliminary results indicate that this enzyme catalyzed only partial dephosphorylation of CoA even when added in great excess. Therefore, despite its other favorable properties we abandoned use of this phosphatase.

Production and analysis of ^{33}P -labeled CoA and CoA thioesters by bacterial DPCKs

As expected, use of $[\gamma\text{-}^{33}\text{P}]\text{ATP}$ as the DPCK substrate resulted in synthesis of radioactive CoA species and each of the CoA thioesters became ^{33}P -labeled (see below). The results obtained using a flow-through scintillation counter to monitor the effluent of the HPLC column; (data not shown) were essentially identical to those obtained by monitoring absorbance (Fig. 3) Since no commercial source of DPCK is available, we next turned to preparation of this reagent. The bifunctional human DPCK/phosphopantetheine adenylyltransferase (PPAT) was difficult to reproducibly purify in an active form and thus we turned to *E. coli* DPCK which is encoded by the *coaE* gene and has been purified and characterized by Mishra et al. [12] and by Nazi et al., [18]. A version of the gene encoding an N-terminally His-tagged version of DPCK was expressed in the standard phage T7 RNA polymerase expression system and the enzyme was readily purified in large quantities. This was also true for the even more robust DPCK encoded by the *aq_1985* open reading frame (Swiss Prot 067792) of the thermophilic bacterium, *A. aeolicus* which was obtained by the same procedure as the *E. coli* protein.

Application of the DPCK method to the CoA metabolite pool of *E. coli*

Since the dephosphorylation and phosphorylation reactions functioned well with pure samples of CoA and each of the short chain CoA thioesters tested, we next tested the method on crude deproteinized samples extracted from *E. coli*. This was done to test the possibility

that other cellular components could inhibit one or both of the two reactions. The samples were deproteinized in order to preclude degradation of the assay reagents by cellular enzymes. The criterion we chose to validate the DPCK assay for use on crude samples extracted from cells was the ability to replicate an established assay. Moreover, the established assay selected also provided an internal standard.

E. coli was chosen as the test organism since by use of appropriate auxotrophic strains, CoA and all of its derivatives can be radioactively labeled by biosynthetic incorporation of a radioactive CoA precursor (which in our case was β -[^3H]alanine) [1;3;10;25]. The cells are first starved for the CoA precursor to deplete the CoA pools and then grown in a chemically defined medium with a radioactive CoA precursor (usually pantothenate or β -alanine) of known specific activity for many generations to ensure uniform labeling of CoA and its derivatives. Such *in vivo* radioactive labeling permits direct quantitation because the specific activity of CoA and its derivatives are the same as that of the labeled CoA precursor added to the medium [1;3;10;25]. This approach thus avoids the potentially artifactual derivatization steps of other protocols. Hence, biosynthetic labeling has been the method of choice for analysis of CoA pools in *E. coli* for over 40 years [1]. In our case biosynthetic labeling was used as an internal standard to test the validity of the new DPCK-based method. If the cellular CoA pools contained compounds that inhibited the dephosphorylation or phosphorylation reactions of our procedure, lower (or perhaps badly skewed) values for CoA and the short chain CoA thioesters relative to those obtained by quantitation of the β -[^3H]alanine labeled compounds would be obtained. Moreover, the DPCK method could be directly compared to the biosynthetic labeling method because the same samples could be analyzed by scintillation counting (the β -particle emissions of ^3H and ^{33}P are readily distinguished). Therefore, the biosynthetic labeling provided a true internal standard for the DPCK method and provided a rigorous test of the validity of our DPCK-based method and its applicability to biological samples.

The biosynthetically-labeled CoA metabolite pool samples were dephosphorylated with SAP and rephosphorylated with DPCK-Ec plus [γ - ^{33}P]ATP. The CoA metabolites of the now doubly labeled samples were then mixed with commercial standards of CoA and the short chain thioesters. This mixture was then fractionated by HPLC and the appropriate fractions (identified by the UV absorbance of the internally added commercial standards) were collected and analysed by dual channel scintillation counting. When the results obtained were converted to molar quantities, the results of the biosynthetic labeling and DPCK methods were essentially identical (< %) for the pool sample analyzed in Fig. 4 and for four other pools extracted from independent cultures of *E. coli* strain SI93 (data not shown). We conclude that the DPCK method can be accurately applied to pools of CoA metabolites isolated from cellular material.

Conclusions

The method we report should be generally applicable to assay of the many structurally diverse short chain CoA thioesters found in nature, although each thioester to be measured should be demonstrated to be quantitatively dephosphorylated by phosphatase treatment and quantitatively rephosphorylated by DPCK. The expression clones for DPCK-Ec and DPCK-Aa are available from the authors and may be freely disseminated.

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Fig. 1.
Flow chart of the assay. Phosphatase is abbreviated as P-ase.

Fig. 2. DPCK is active with CoA esters. Panel A. The reactions used to detect DPCK activity. The abbreviations are PK, pyruvate kinase and LDH, lactate dehydrogenase. Panel B. Activity of DPCKH with dephosphosphorylated derivatives of CoA and CoA esters. The substrates, dephospho-CoA, dephospho-acetyl-CoA, dephospho-succinyl-CoA, and dephospho-malonyl-CoA were generated by shrimp alkaline phosphatase (SAP) reactions and were present in approximately equimolar amounts. Reactions mixtures containing all of the assay components except that the coupling enzymes were incubated until a stable baseline had been reached (~80 seconds) at which time the coupling enzyme mixture was added to start the reaction. The decrease in absorbance at 340 nm with time indicates the oxidation of NADH dependent on conversion of the dephospho-CoA to the phosphorylated product. Starting the reactions by addition of DPCKH gave similar results.

Figure 3.

HPLC separation of CoA, CoA esters and their dephosphorylated derivatives. The CoA and CoA esters were separately dephosphorylated then a portion of each compound was re-phosphorylated with DPCK-*Hs* as per the standard reaction conditions. The products of both the dephosphorylation and re-phosphorylation reactions were chromatographed on a C18 HPLC column as described previously [10]. The absorbance at 254 nm was measured over time. The dashed lines represent the dephospho-CoA or dephospho-CoA ester standards whereas the solid line denotes the product of phosphorylation of that compound. Panel A, CoA; panel B, acetyl-CoA; panel C, malonyl-CoA and panel D, succinyl-CoA. The peak eluting at ~5 minutes is ATP remaining from the re-phosphorylation reaction.

Figure 4.

Comparative Analysis of CoA and CoA esters radiolabeled both *in vivo* and *in vitro*. The CoA pools were extracted from strain SI192 after overnight growth in minimal medium supplemented with β -[3- ^3H]alanine and the extracted compounds were dephosphorylated and subsequently re-phosphorylated using [γ - ^{33}P]ATP as described in Materials and Methods. The reaction mixtures were then separated via HPLC [10]. The CoA and CoA ester peaks were collected and the levels of ^3H and ^{33}P were determined by dual channel scintillation counting. The molar ratios of the ^{33}P -labeled to ^3H -labeled compounds varied from 0.91 to 1.04 among five separate dual label experiments.