Iso-Coenzyme A

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Running Title: Iso-Coenzyme A
Summary

Iso-coenzyme A (iso-CoA) is an isomer of coenzyme A (CoA) in which the monophosphate is attached to the 2’-carbon of the ribose ring. While iso-CoA was first reported in 1959 by Khorana and coworkers to be a byproduct of the chemical synthesis of CoA, relatively little literature attention has been focused on iso-CoA or on acyl-iso-CoA compounds. We now report structural characterizations of iso-CoA, acetyl-iso-CoA, acetoacetyl-iso-CoA, and β-hydroxybutyryl-iso-CoA using MS, MS/MS, and homo- and hetero-nuclear NMR analyses. While the 2’-phosphate isomer of malonyl-CoA was recently identified in commercial samples, previous characterizations of iso-CoA itself have been based on chromatographic analyses, which ultimately rest on comparisons with the degradation products of CoA and NADPH, or have been based on assumptions regarding enzyme specificity. We describe HPLC methodology to separate the isomers of several CoA-containing compounds. We also report here the first examples of iso-CoA-containing compounds acting as substrates in enzymatic acyl-transfer reactions. Finally, we describe a simple synthesis of iso-CoA from CoA which utilizes β-cyclodextrin to produce iso-CoA with high regioselectivity, and we demonstrate a plausible mechanism which accounts for the existence of iso-CoA isomers in commercial preparations of CoA-containing compounds. We anticipate that these results will provide methodology and impetus for investigating iso-CoA compounds as potential pseudosubstrates or inhibitors of the more than 350 known CoA-utilizing enzymes.
Introduction

Iso-coenzyme A (iso-CoA) is an isomer of coenzyme A (CoA) in which the monophosphate is attached to the 2’-carbon of the ribose ring. Iso-CoA was first reported in 1959 by Khorana and coworkers (1,2) to be a byproduct of the chemical synthesis of CoA. The final step of this synthesis entailed acid-catalyzed hydrolysis of cyclic-coenzyme A (Scheme 1), to produce a 50:50 mixture of two products which were separated using ECTEOLA-cellulose ion exchange chromatography (1,2). Direct structural analysis was not readily available in 1959; consequently, the authors deduced the structure of iso-CoA by establishing that, in contrast to CoA, iso-CoA was not a substrate for the enzyme phosphotransacetylase (1,2). Khorana and coworkers also used paper chromatography of phosphodiesterase

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1The abbreviations used are:

Iso-CoA, iso-Coenzyme A; CoA, coenzyme A; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; TBAHS, tetrabutyl ammonium hydrogen sulfate; TCEP, triscarboxyethyl phosphine; MS, mass spectrometry; MS/MS, tandem mass spectrometry; MRM, Multiple Reaction Monitoring; AcCoA, acetyl-coenzyme A; AcAcCoA, acetoacetyl-coenzyme A; HBCoA, (β-hydroxybutyryl-coenzyme A; PHB, poly-(β)-hydroxybutyric acid; RP-HPLC, Reversed Phase High Performance Liquid Chromatography; HMQC, Heteronuclear Multiple Quantum Correlation; NMR, Nuclear Magnetic Resonance; COSY, Correlation Spectroscopy; EPPS, 4-(2-Hydroxyethyl)piperazine-1-propanesulfonic acid.
hydrolysates to show that enzymatic hydrolysis of iso-CoA produces adenosine-2',5'-diphosphate, exclusively (1,2). Since Khorana’s original work, four additional chemical syntheses of CoA have been reported (3-8) all of which produce cyclic-CoA as the penultimate product; cyclic-CoA is then hydrolyzed either chemically, to produce a mixture of CoA and iso-CoA (1,2,4-6,8), or enzymatically with ribonuclease T2 to produce CoA regioselectively (3-7).

Recently, Minkler et al. (9) observed the 2'-phosphate isomer of malonyl-CoA in commercial samples. These authors reported that HPLC-purified samples of each isomer exhibited identical UV, HPLC-MS, and HPLC-MS/MS properties, and the identity of the 2'-phosphate isomer was convincingly demonstrated by comparing the 1D-NMR spectra to that of 2',5'-ADP. In related work, Retey and coworkers have synthesized several dethia-CoA analogs (10-15), in which the sulfur of CoA is replaced by a methylene, as mixtures of the 2'- and 3'-phosphate isomers; two of these analogs were purified to homogeneity and characterized by 1D ¹H-NMR, ³¹P-NMR, and in one case FAB-MS (13,14). Dethia-CoA analogs were also synthesized and characterized by Stewart and coworkers (16-18); unfortunately, they were unable to separate the 2'- and 3'-phosphate isomers and the NMR data could only be obtained on isomeric mixtures. (17) However, to the best of our knowledge, no full structural characterization has ever been reported in the literature for iso-CoA, acetyl-iso-CoA, acetoacetyl-iso-CoA, or β-hydroxybutyryl-iso-CoA.

The issue of whether or not enzymes which act upon CoA-containing substrates discriminate between the 2'- and 3'-phosphate isomers is
of obvious relevance to the design of new classes of inhibitors and pseudosubstrates. As mentioned above, in Khorana’s early work the enzyme phosphotransacetylase was reported to be unreactive towards iso-CoA (1,2). Similarly, Retey and coworkers have reported that FAD-dependent isobutanoyl-CoA dehydrogenase is reactive with isobutanoyl-dethia-CoA but not with the iso-CoA analog (13), and methylmalonyl-CoA pyruvate carboxylase also distinguishes between the isomers, reacting exclusively with propionyl-dethia-CoA (10). In contrast, Thorpe et al. (18) reported that substrate mixtures containing both dethia-iso-CoA and dethia-CoA derivatives are capable of reducing enzyme bound FAD. Likewise, Rossier (19) reported that choline acetyltransferase is inhibited by both seleno-CoA and seleno-iso-CoA and Wagner et al. (14) have reported that N-myristoyltransferase is competitively inhibited by both carbadethia-CoA and carbadethia-iso-CoA, although in both cases the iso-CoA analogs are less potent. In the cases of citrate synthase and carnitine palmitoyltransferase (17) it is difficult to draw a clear conclusion from the data since the experiments were performed with mixtures containing both the 2’- and 3’-isomers. Clearly this issue needs to be reexamined for many CoA-utilizing enzymes using purified and characterized iso-CoA substrate analogs.

Herein we report structural characterizations of iso-CoA, acetyl-iso-CoA, acetoacetyl-iso-CoA, and β-hydroxybutyryl-iso-CoA. Historically, characterizations of the structure of iso-CoA have been based on chromatographic analyses (1,2), which ultimately rest on comparisons with the degradation products of CoA and NADPH (20-22), or have been based on assumptions regarding enzyme specificity (5,6,8).
We describe HPLC methodology to separate the isomers of several CoA-containing compounds, and the characterization of iso-CoA structures using MS, MS/MS, and NMR analyses. We also report here the first examples of iso-CoA-containing compounds acting as substrates in enzymatic acyl-transfer reactions. Finally, we describe a simple synthesis of iso-CoA from CoA which utilizes β-cyclodextrin to produce iso-CoA with high regioselectivity, and we demonstrate a plausible mechanism which accounts for the existence of iso-CoA isomers in commercial preparations of CoA-containing compounds.

**Experimental Procedures**

*Materials.* The lithium salts of CoA (93% purity) purified from yeast, acetyl-CoA (93% purity), β-hydroxybutyryl-CoA (99% purity), the sodium salt of acetoacetyl-CoA (90% purity), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), β-cyclodextrin, and tetrabutyl ammonium hydrogen sulfate (TBAHS) were purchased from Sigma (St. Louis, MO). Triscarboxyethyl phosphine (TCEP) was from Molecular Probes (Eugene, OR). All HPLC or Optima Grade solvents and all other compounds were obtained from Fisher Scientific.

*HPLC Mass Spectrometry.* Commercial CoA and acetyl-CoA standards were analyzed with a Micromass Quattro LC triple quadrupole tandem mass spectrometer equipped with an ESI source connected to an Hewlett-Packard series 1100 HPLC system. Resolution of the isomers was achieved with an Agilent Hypersil AA-ODS 2.3 x 200 mm column attached.
to a Phenomenex SecurityGuard™ (C18, 4 mm length x 3.0 mm I.D.), using an isocratic mobile phase of 96% 200 mM ammonium acetate pH 6.0: 4% acetonitrile at a flow rate of 0.2 mL/min. ESI mass spectra were obtained in the positive ion mode with nitrogen used as the nebulizer and desolvation gas at flow rates of approximately 80 and 580 L/h, respectively. The cone voltage was set to 50 V and the capillary voltage to 3.5 kV. Tandem mass spectrometry (MS/MS) using multiple reaction monitoring (MRM) was performed on the (M+H)+ ions of the isomers of CoA (m/z 768), acetyl-coA (m/z 810), acetoacetyl-CoA (m/z 852), and β-hydroxybutyryl-CoA (m/z 854) using argon as the collision gas at a collision energy of 20 eV for the collision induced dissociation of the precursor ion. The commercial standards were monitored at the following transitions: CoA at m/z’s 768Æ159, 768Æ261, and 768Æ428, and acetyl-CoA at m/z 810Æ303 and m/z 810Æ428, and acetoacetyl-CoA at m/z’s 852Æ136, 852Æ243, 852Æ261, 852Æ345, and 852Æ428, and β-hydroxybutyryl-CoA at m/z’s 854Æ136, 854Æ245, 854Æ347, and 854Æ428.

High Resolution ESI MS of HPLC-Purified Samples. Exact mass and MS/MS analyses were performed using HPLC-purified samples of CoA and the isomer on an Applied Biosystems QSTAR XL hybrid quadrupole-TOF LC/MS/MS mass spectrometer. The QSTAR XL was operated in the positive ion mode using a needle voltage of 5500 V. MS analysis was performed over a mass range of m/z 310-1500 and MS/MS spectra of the m/z 768 precursor ion were obtained over the mass range of m/z 50-800.

NMR Analyses. NMR spectra of iso-CoA were obtained in D2O in a 5 mm sample tube on a Bruker DRX 500 spectrometer at 500.13 mHz for 1H
and 202.46 mHz for $^{31}$P using an inverse triple resonance probe maintained at 298 K. The COSY experiment was performed using the standard Bruker program cosyprqf, with presaturation during the 2 s relaxation delay on the HOD signal; 2048 data points in the F2 dimension and 128 increments in F1. The data matrix was processed to give a matrix of 1024 x 1024 points, and a sine-bell apodization function was applied before Fourier transformation.

The parameters for the two-dimensional heteronuclear multiple quantum correlation ($^1$H–$^{31}$P HMQC) experiment are as follows: hmqcqf standard Bruker program, 1.5 s recycle delay, 1024 data points in F2 and 128 increments in F1, 8 Hz coupling constant, GARP $^{31}$P decoupling during acquisition; shifted sine-squared apodization before fourier transform. The chemical shift assignments for the HPLC-purified CoA and iso-CoA are labeled above their respective peaks in the proton spectra. The few unlabeled peaks were either due to the iso-CoA disulfide formed during purification and analysis in the absence of reducing agents, or to an unknown impurity. Coupling constants were obtained through successive decoupling experiments, and the unresolved coupling constants were then obtained by simulating the spectra using the gNMR (23) and MestRe-C (24) software packages.

**HPLC.** HPLC analyses of CoA, acetyl-CoA, acetoacetyl-CoA and β-hydroxybutyryl-CoA and the isomers were performed on a Waters LC Module I Plus HPLC system with auto-injection of samples using the Millennium Chromatography Manager Software (Waters, Milford, MA, USA). A Waters NovaPak C18 Reverse Phase column, 4 µm particle size, 150 mm x 3.9 mm I.D. was used with a Phenomenex SecurityGuard™ containing a
C18, 4 mm length x 3.0 mm I.D., guard cartridge. Isocratic analyses were performed at a wavelength of 261 nm with a mobile phase containing 96% of 200 mM ammonium acetate pH 6.0: 4% acetonitrile at a flow rate of 1.0 mL/min. Semi-Prep HPLC-Purification was performed on an Allsphere ODS-2 semi-prep column, 250 mm x 10 mm I.D. with a Phenomenex SecurityGuardTM containing a C18, 4 mm length x 3.0 mm I.D., guard cartridge using isocratic elution with a mobile phase of 97.5% 200 mM ammonium acetate pH 6.0: 2.5% acetonitrile at 7 mL/min.

Two peaks for each CoA-containing sample were observed with four different RP-HPLC columns, and three different HPLC systems using both UV-Vis and MS detection. HPLC-purified samples of CoA were stable in neutral solution and iso-CoA was not generated in deionized water, at pH 3 or at pH 11 (data not shown).

Enzymatic Analyses of Iso-CoA and Acyl-iso-CoA Compounds. The enzymes β-ketothiolase (E.C. 2.3.1.16), acetoacetyl-CoA reductase (E.C. 1.1.1.36), and PHB synthase (E.C. currently not classified) which all utilize CoA-containing substrates were examined for the ability to react with the respective iso-CoA-containing analogs. Enzymatic reactions were carried out in a solution of 16µM acetyl-CoA isomers, 1.0 mM NADPH and 5mM TCEP in 150 mM EPPS at a final pH of 7.8 and 37 °C. The first reaction was initiated by addition of β-ketothiolase and acetoacetyl-CoA reductase, and the second reaction with PHB synthase. Enzyme reactions were quenched with 0.27 M ice-cold perchloric acid followed by centrifugation prior to HPLC analysis as described above.
Regioselective Synthesis of Iso-CoA (III)

CoA Disulfide (CoASSCoA). HPLC-purified CoA (II), 10 mg (13 mM), was dissolved in 1 mL of 20 mM ammonium carbonate pH 8.5, reacted overnight at 37 °C, and then lyophilized to produce CoA-disulfide with 100% yield by HPLC. The reaction progress was monitored via HPLC using the HPLC procedure described above except the mobile phase contained 6% acetonitrile.

Cyclic-CoA-disulfide. The CoA-disulfide lyophile was dissolved in 1 mL of 20 mM MES pH 6.0, 10 mg (52 mM) of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was added and the pH readjusted. Reaction progress was monitored by HPLC after rapidly reducing an aliquot of the disulfide to the free thiol with TCEP at pH 8.0 for 2 min. When the reaction was complete, the entire mixture was lyophilized. The lyophile was dissolved in 200 mM ammonium acetate pH 6.0 buffer and the entire solution applied to a 500 mg Supelco C8 solid phase extraction cartridge that was pre-equilibrated with the same buffer. Residual EDC and the urea byproduct were eluted from the cartridge with the equilibration buffer, and the cyclic-CoA-disulfide was eluted in ca. 5 mL with 90% 200 mM ammonium acetate pH 6.0: 10% acetonitrile. The cyclic-CoA-containing fractions were pooled and lyophilized.

Regioselective Synthesis of Iso-CoA (III). The cyclic-CoA-disulfide lyophile was dissolved in 160 mL of 0.05 mM bicarbonate buffer (I=0.01) pH 9.5 containing 3 M KCl and 15 mM β-cyclodextrin and the pH readjusted. The solution was stirred at 30°C, with the pH maintained at 9.5, for approximately 5 days or until the reaction was
deemed complete by HPLC, at which point the pH was adjusted to 3.0 to quench the reaction. This provided a final solution of iso-CoA with 83% regioselectivity by HPLC. Acetone precipitation and filtration served to remove some of the KCl and β-cyclodextrin from the iso-CoA-containing solution and permitted rotary evaporation followed by vacuum evaporation to dryness. Residual β-cyclodextrin was removed by repeated trituration with DMF and the remaining solid dried with ethyl ether. The final solid was then dissolved in 200 mM ammonium acetate pH 6.0, the pH readjusted, and the solution desalted by passing through a 500 mg Supelco C8 solid phase extraction cartridge. The desalted isomeric mixture of 83% iso-CoA: 17% CoA was eluted as previously described and the desalted mixture lyophilized. Final purification of iso-CoA was performed by dissolving the desalted lyophile in a minimal quantity of 5 mM TCEP in EPPS pH 8.0, to reduce the CoA-disulfide to the free thiol, subjected to semi-prep HPLC as previously described, and lyophilized.

\textbf{Acid Catalyzed Synthesis of Iso-CoA (III).} HPLC-purified CoA (II), 1.6 mg, was dissolved in 0.5 mL of 0.5 M HCl, and the reaction was monitored by HPLC at room temperature until equilibrium was reached, which generated a mixture of 60% CoA and 40% iso-CoA. The identity of iso-CoA produced in this manner was confirmed by NMR analysis after HPLC purification and lyophilization, as described previously.
Results

Mass Spectrometric Structural Characterization of CoA and Acetyl-CoA Samples. The HPLC-MS and HPLC-MS/MS chromatograms obtained for commercial CoA samples are shown in panel A of Figure 1. The left portion of panel A shows an HPLC-MS chromatogram of a commercial CoA sample, which exhibits 2 peaks with identical (M+H)^+ ions at m/z 768 suggesting that commercial CoA samples contain two compounds with identical masses. Therefore, HPLC-MS/MS experiments using multiple reaction monitoring (MRM) were carried out to garner further structural information. It is evident from the right portion of panel A that the CoA samples exhibit two peaks for the MRM transitions m/z 768→159, m/z 768→261, and m/z 768→428. Thus, these HPLC-MS and HPLC-MS/MS results suggest the presence of isomeric compounds with identical fragmentation patterns. To confirm these analyses, each peak in the commercial CoA sample was purified by preparative HPLC, and high resolution exact mass and MS/MS analyses were performed individually on each isomer. As shown in Figure 1 Panel B Inserts, the two HPLC-purified samples exhibited identical exact masses (M+H)^+ of 768.1264 with an error of 5.09 ppm with respect to the calculated molecular mass. Additionally, the MS/MS (precursor ion m/z 768) spectra for the two HPLC-purified samples depict nearly identical fragmentation patterns, which exhibit only minor variations in the relative populations of ions at m/z 136, m/z 159, and m/z 330. These results unequivocally confirm the presence of isomeric forms of CoA in the commercial material, and the MS/MS results showing that these isomers exhibit virtually identical fragmentation patterns indicate
that these isomers have extremely similar structures. HPLC-MS and HPLC-MS/MS experiments were also carried out with commercial acetyl-CoA, acetoacetyl-CoA and β-hydroxybutyryl-CoA (see supplemental material); these experiments also revealed the presence of two isomeric compounds with identical (M+H)+ ions that exhibited identical fragments by HPLC-MS/MS using MRM at the observed transitions.

Structural Characterization of Iso-CoA by NMR. 1D 1H-NMR spectra for the two HPLC-purified isomers of CoA are shown in Panels A and B of Figure 2. The spectrum in Panel A, for the earlier-eluting isomer, is very similar to several previously-published CoA spectra (25-30), and the chemical shift assignments were made according to d’Ordine et al (29). In contrast, the spectrum shown in Panel B, for the later-eluting isomer, is very different in the 4-5 ppm region. Therefore, 2D 1H-1H COSY experiments (Figure 3) were obtained for this isomer in order to assign the chemical shifts. The chemical shift assignments and coupling constants are listed in Table 1. It is evident that for the later-eluting isomer the 2'-proton is shifted downfield by approximately 0.20 ppm while the 3'- and 4'-protons are respectively shifted up field by 0.15 and 0.20 ppm, and the two 5'-protons have significantly different coupling patterns. Since it seemed likely that the position of the phosphate on the ribose ring gives rise to these differences, 1H-31P HMQC experiments were carried out to explicitly assign the position of each phosphate. As shown in Figure 4, the 1H-31P HMQC spectra for the later-eluting compound, unequivocally establishes that the monophosphate is attached at the
2’-carbon, thus designating this compound as iso-CoA. HPLC experiments, presented in Figure 5, established that commercial samples of CoA, acetyl-CoA, acetoacetyl-CoA and β-hydroxybutyryl-CoA designated as 93%, 93%, 90%, and 99% pure contain approximately 10%, 15%, 18% and 3% of the 2’-monophosphate isomers, respectively.

Iso-CoA-containing Compounds are Substrates for β-ketothiolase, Acetoacetyl-CoA Reductase and PHB Synthase. The reaction time courses shown in Figure 6 for CoA, acetyl-CoA, and β-hydroxybutyryl-CoA and their respective iso-CoA isomers demonstrate that the three enzymes, β-ketothiolase, acetoacetyl-CoA reductase, and PHB synthase, can catalyze reactions with either isomer. These data represent the first examples of iso-CoA-containing compounds acting as substrates in enzymatic acyl-transfer reactions. In the coupled two-enzyme system in Panel A, β-ketothiolase condenses two molecules of acetyl-CoA to acetoacetyl-CoA, which is immediately reduced to β-hydroxybutyryl-CoA by the second enzyme acetoacetyl-CoA reductase. In Panel B a third enzyme, PHB synthase, has been added which converts the β-hydroxybutyryl-CoA to poly-(β)-hydroxybutyric acid. It is evident from the time courses in both panels that in all cases both the CoA- and iso-CoA isomers are processed with equal facility. In other experiments, we find that acetoacetyl-CoA and its iso-CoA isomer react with equal facility in the back reaction to produce the acetyl-CoA product (data not shown).
Regioselective Synthesis of Iso-CoA. Since iso-CoA and acyl-iso-CoA compounds may be useful as potential inhibitors or pseudosubstrates, a synthesis capable of producing high yields of the 2’-phosphate isomer is desirable; to this end, we report a simple synthesis of iso-CoA from CoA. As shown in Scheme 2, CoA was initially converted to the dimeric disulfide, and the water soluble coupling agent 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was then used to form the 2’,3’-cyclic-CoA intermediate in a reaction analogous to the synthesis of 2’,3’-cyclic-NADP+ (31). We note that the first steps of the synthesis are carried out using the CoA dimer to avoid possible quenching of the EDC; the dimer is reduced back CoA in Step 3. Finally, base-catalyzed hydrolysis is carried out in the presence of β-cyclodextrin in high salt at pH 9.5 to produce iso-CoA with a 83% yield.

Acid-Catalyzed Phosphate Migration. We investigated the acid-catalyzed migration of the CoA monophosphate group in an attempt to furnish a plausible mechanism for the existence of the 2’-phosphate isomers in commercial preparations of CoA-containing compounds. In these experiments HPLC-purified CoA was incubated in the presence of 0.5 M HCl, and the relative amounts of CoA and iso-CoA were determined by HPLC as a function of time. As shown in Figure 7, a final equilibrium mixture of 60% CoA and 40% iso-CoA was attained within 60 hours. We note that approximately 10% iso-CoA is present after only 4 hours, so the iso-CoA content of commercial preparations may indeed be produced in this fashion during the processing of the product.
Discussion

The vital role of coenzyme A in metabolism is underscored by the sheer quantity of classified enzymes (32,33) that react with CoA-containing molecules; we count greater than 9% of the total known enzymes to be of this type. The 2′-phosphate isomer of CoA, first named iso-CoA in Khorana’s 1959 report (1,2), has traditionally been considered an undesirable synthetic byproduct or has been simply ignored. Indeed, a review of the literature reveals that only eight of the more than 350 CoA-utilizing enzymes (32) have been examined for the ability to discriminate between the 2′- and 3′-phosphate isomers (1,2,10,13,14,17-19,34), and in two of these cases the enzymes have been shown to accept purified 2′-phosphate isomers as substrates or inhibitors (14,19). Moreover, the majority of the compounds examined in these eight cases are synthetic analogs of CoA, such as the dethia- or seleno-derivatives, and not iso-CoA itself.

In the results presented here, CoA and iso-CoA were purified by HPLC, and high resolution exact mass MS and MS/MS analyses unequivocally established that the compounds are constitutional isomers with extremely similar fragmentation patterns. Direct structural identification of the two HPLC-purified isomers was then performed using ¹H-NMR, ¹H-¹H COSY, and ¹H-³¹P HMQC, and the results unequivocally established that the monophosphate of the iso-CoA isomer is attached to the 2′-carbon of the ribose ring. Structural identification of acetyl-iso-CoA, acetoacetyl-iso-CoA and β-hydroxybutyryl-iso-CoA was also carried out using HPLC-MS and HPLC-MS/MS analyses.
We report here the first example of iso-CoA-containing compounds acting as substrates in acyl-transfer reactions. Three enzymes, β-ketothiolase, acetoacetyl-CoA reductase, and PHB synthase, successfully react with the 2′-isomers of their natural substrates, and the reaction time courses indicate that all three enzymes react with either isomer with equal facility. These results suggest that other enzymes should be examined for their reactivities towards the 2′-phosphate isomers of their CoA-containing substrates. We note that approximately 40 enzymes have been evaluated for their abilities to interact with dephospho-CoA analogs. In the case of phosphotransacetylase, Iyer and Ferry (35) have identified a salt bridge at the binding site of this enzyme, which they have suggested imparts a 350-fold preference for CoA over dephospho-CoA; a salt bridge between an arginine and the 3′-phosphate moiety of CoA has also been reported for choline acetyltransferase (36,37). This type of salt-bridge formation may be a general feature of enzymes that react with CoA-containing substrates. If so, it remains to be determined whether this binding interaction would be distorted by the presence of a phosphate at the 2′-position, rather than the 3′-position, of the ribose ring.

The traditional method for resolving isomers of CoA-containing compounds, ion exchange chromatography (1,2,14,34), is often lengthy, does not achieve baseline resolution, and thus may have hampered investigations into isomers of CoA (19). We report here efficient HPLC methodology which achieves baseline resolution of CoA and acyl-CoA isomers. This technique, which improves upon earlier work by
Norwood et al. (38), is the first HPLC method to separate a series of iso-CoA compounds, and we are able to separate four iso-CoA isomers within 30 minutes with baseline resolution using convenient isocratic elution. We note that inspection of published HPLC elution profiles of CoA-containing compounds reveal several chromatograms which appear to contain iso-CoA compounds that were not identified as such by the authors (39-43). Furthermore, iso-CoA isoforms may have been overlooked by previous authors because the isomers will coelute under stronger HPLC elution conditions.

We also report here the first regioselective chemical synthesis of iso-CoA from CoA. Previously reported methods which produce iso-CoA compounds as a byproduct of cyclic-CoA hydrolysis yield mixtures containing both the 2'- and 3'-phosphate isomers in ratios of approximately 40% : 60%, respectively (1,2,5,6,8). In our simple synthesis, the cyclic-CoA moiety is efficiently produced from CoA, and then base-catalyzed hydrolysis in the presence of β-cyclodextrin in high salt produces iso-CoA regioselectively in high yields. Our results are in agreement with those of Komiyama and coworkers (44,45), who previously reported that β-cyclodextrin-based hydrolysies of adenosine and guanosine 2',3'-cyclic phosphate compounds give reversed regioselectivity (i.e. 3'-cleavage) as compared to enzymatic hydrolysis by ribonuclease. These authors theorize that the adenine moiety forms an inclusion complex with β-cyclodextrin, which generates steric constraints that direct hydrolysis toward the 3'-bond, producing the 2'-phosphate species, regioselectively.
We propose acid-catalysis as a plausible mechanism to account for the existence of the 2′-phosphate isomers in commercial preparations of CoA-containing compounds. In 1954, Kaplan and coworkers published preliminary reports about the acid-catalyzed synthesis of iso-CoA from CoA (21,46); however, these authors did not prove iso-CoA formation but only demonstrated that exposing CoA to strong acid decreased specific 3′-nucleotidase activity. Nevertheless, the acid-catalyzed migration of the 2′- and 3′-phosphate groups of NADPH (47) and nucleotides (48-51) is well established in the literature. In our hands, treatment of CoA with 0.5 M HCl produces an equilibrium mixture of 40% iso-CoA and 60% CoA within 60 hours, and 10% of the isomer can be generated within 4 hours. These results indicate that acid catalysis is a viable mechanism for rapidly producing iso-CoA from CoA, especially considering that strong acids are often used in several published CoA isolation procedures (52-56).

The biological significance of iso-CoA compounds is currently unknown. Khorana’s original report (1,2) stating that phosphotransacetylase does not react with iso-CoA probably has led to the generally held contention that the 2′-isomers are unnatural compounds. In contrast, Kurooka et al. (57) suggested that iso-CoA might be formed from crude cell extracts of Proteus mirabilis in the presence of 3′-dephospho-CoA. Similarly, Michaelson (3) reported that an unnamed partially-purified enzyme from calf brain selectively generated iso-CoA from cyclic-CoA in vitro. This enzyme was probably 2′,3′-cyclic 3′-phosphodiesterase, which selectively produces 2′-phosphate nucleosides from their 2′,3′-cyclic species—such as NADP+.
generation from cyclic-NADP⁺ (31). Although this enzyme constitutes approximately 4% of all myelin protein, to date the actual in vivo substrate for this enzyme’s esterase activity has not been established (58-60). These reports suggest that iso-CoA could possibly be generated enzymatically in vivo from either dephospho-CoA or 2′,3′-cyclic-CoA; however, no definitive evidence for this notion yet exists.

While the biological significance of iso-CoA is presently unknown, the potential for this class of isomeric compounds is clearly significant. Iso-CoA isoforms have potential as pseudosubstrates, inhibitors, and as probes for investigating CoA binding to the extensive class of CoA-utilizing enzymes. We anticipate that our investigation, in addition to generating the first reports of enzymes performing acyl-transfer reactions with iso-CoA isomers, will provide the necessary methodology for future studies into iso-CoA and acyl-iso-CoA compounds.

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**Figure Legends**

**Scheme 1: Final Reaction in the Total Synthesis of CoA.**
Chemical hydrolysis of cyclic-CoA (I) produces two products, CoA (II) and iso-CoA (III). Carbons are labeled according to d’Ordine et al (29).

**Scheme 2: Regioselective Synthesis of Iso-CoA from CoA.** This is the first regioselective chemical synthesis of iso-CoA reported, and utilizes β-cyclodextrin in high salt to confer iso-CoA regioselectively in high yields.

**Figure 1: Mass Spectrometric Analysis of Commercial CoA Samples.**
The left portion of Panel A shows the HPLC-MS extracted ion chromatogram for a commercial CoA sample which exhibited two peaks with identical (M+H)^+ ions, m/z 768. On the right are the HPLC-MS/MS chromatograms observed at the labeled MRM transitions m/z 768→159, m/z 768→261, and m/z 768→428. Subsequent NMR analysis established that the earlier-eluting compound, 11.1 min, is authentic CoA and the later-eluting compound, 13.8 min, is iso-CoA. Putative fragmentation patterns are indicated in the center. The commercial CoA sample was then purified by preparative HPLC and, as shown in Panel B, each peak was subjected to high resolution ESI-MS (Inserts) and MS/MS analyses. Authentic CoA is shown on the left of the panel, and iso-CoA is depicted on the right. The inserts are the exact mass spectra for each HPLC-purified isomer which exhibited an m/z 768.1264 with an error of 5.09 ppm with respect to the calculated molecular mass of CoA.
Figure 2: $^1$H-NMR of HPLC-purified Samples of CoA and Iso-CoA. 
HPLC-purified samples of CoA and iso-CoA analyzed by $^1$H-NMR, with labels corresponding to the numbering scheme shown in Scheme 1.

Figure 3: $^1$H-1H COSY of Iso-CoA. The $^1$H-1H COSY of HPLC-purified iso-CoA unequivocally assigns the proton chemical shifts of the ribose ring. The inserted marker lines illustrate how the indicative chemical shift of the anomeric proton, labeled at 6.18 ppm, was used to determine the position of each proton in the ribose ring.

Figure 4: $^1$H-$^{31}$P HMQC of Iso-CoA. The $^1$H-$^{31}$P HMQC for the HPLC-purified isomer unequivocally demonstrates that the monophosphate, located at -0.9 ppm, clearly corresponds to the 2'-proton of the ribose ring; thus this isomer is designated as iso-CoA. Additionally, since the two phosphates of the pyrophosphate linkage couple with the protons on the 5' and 1'' carbons, no other phosphate rearrangements are evident.

Figure 5: HPLC of Four Commercial CoA-containing Samples. The HPLC elution profile of commercial CoA, acetyl-CoA, acetoacetyl-CoA, and β-hydroxybutyryl-CoA exhibits two peaks for each compound and have the following retention times and purities: 4.25 min., CoA, 90%; 5.5 min., iso-CoA, 10%; 12.75 min., AcCoA, acetyl-CoA, 85%; 14.5 min., AcAcCoA, acetoacetyl-CoA, 82%; 17.1 min., Iso-AcCoA, acetyl-iso-CoA, 15%; 18.25 min., HBCoA, β-hydroxybutyryl-CoA, 97%; 19.5 min., Iso-AcAcCoA, acetoacetyl-iso-CoA, 18%; 24.6 min., Iso-HBCoA, β-hydroxybutyryl-iso-CoA, 3%.
Figure 6: The First Example of Enzymatic Acyl-transfer Reactions Occurring with 2′-isomers of CoA-containing Compounds. Enzymatic reaction time courses for the enzymes β-ketothiolase, acetoacetyl-CoA reductase, and PHB synthase indicate that these enzymes catalyze identical reactions exhibiting equivalent kinetic rates with both 2′- and 3′-isomers of acetyl-CoA, CoA, and β-hydroxybutyryl-CoA. Panel A: β-ketothiolase and acetoacetyl-CoA reductase are added to a solution containing acetyl-CoA and its isomer, NADPH, and TCEP buffered at pH 7.8. This coupled-enzyme reaction initiates the consumption of two molecules of acetyl-CoA or acetyl-iso-CoA; thus, generating one molecule of CoA or iso-CoA and one molecule of β-hydroxybutyryl-CoA or the isomer. Panel B: Addition of PHB synthase to the previous coupled-enzyme system (Panel A) results in the conversion of β-hydroxybutyryl-CoA and its isomer to poly-β-hydroxybutyric acid; this irreversible polymerization stimulates the consumption of further acetyl-CoA isomers with CoA or iso-CoA liberation during both processes. Acetoacetyl-CoA, the product of the β-ketothiolase mediated condensation of 2 acetyl-CoA molecules, is below the limit of detection by HPLC. ■ AcCoA, acetyl-CoA; Δ Iso-AcCoA, acetyl-iso-CoA; ■ CoA; ○ Iso-CoA; ♦ HBCoA, β-hydroxybutyryl-CoA; □ Iso-HBCoA, β-hydroxybutyryl-iso-CoA.

Figure 7: Acid-catalyzed Synthesis of Iso-CoA HPLC-purified CoA was incubated in the presence of 0.5 M HCl, and the relative quantities of ■ CoA and ♦ iso-CoA were monitored by HPLC as described in the text.
Table 1  Iso-CoA $^1$H- and $^{31}$P-NMR chemical shift assignments and coupling constants.

| $^1$H Chemical Shift | Proton Position | Integration | Multiplicity | J (MHz) |
|----------------------|-----------------|-------------|--------------|---------|
| 8.41                 | 8               | 1           | s            |         |
| 8.18                 | 2               | 1           | s            |         |
| 6.18                 | 1'              | 1           | d            | J$_{1'-2'}$ 5.15 |
| 4.98                 | 2'              | 1           | m            | J$_{2'-3'}$ 5.09 |
|                     |                 |             |              | J$_{2'-p2'}$ 8.69 |
| 4.56                 | 3'              | 1           | t            | J$_{3'-4'}$ 4.56 |
| 4.32                 | 4'              | 1           | m            | J$_{4'-5'a}$ 3.92 |
|                     |                 |             |              | J$_{4'-5'b}$ 2.83 |
|                     |                 |             |              | J$_{4'-p5'}$ 2.00 |
| 4.18                 | 5'              | 1           | m            | J$_{5'_{b-p5'}}$ 4.58 |
| 4.13                 | 5'              | 1           | m            | J$_{5'_{a-p5'}}$ 5.08 |
|                     |                 |             |              | J$_{5'_{a-5'b}}$ 11.63 |
| 3.92                 | 3''             | 1           | s            |         |
| 3.73                 | 1''             | 1           | dd           | J$_{1''-1'}$ 9.90 |
|                     |                 |             |              | J$_{1''-p1''}$ 5.09 |
| 3.48                 | 1''             | 1           | dd           | J$_{1''-p1''}$ 4.83 |
| 3.38                 | 5''             | 2           | t            | J$_{5''-6''}$ 6.53 |
| 3.24                 | 8''             | 2           | t            | J$_{8''-J9''}$ 6.59 |
| 2.54                 | 9''             | 2           | t            |         |
| 2.37                 | 6''             | 2           | t            |         |
| 0.89                 | 10''            | 3           | s            |         |
| 0.67                 | 11''            | 3           | s            |         |

| $^{31}$P Chemical Shift | Phosphate Position | Integration | Multiplicity |
|------------------------|-------------------|-------------|--------------|
| -0.90                  | 2'                | 1           | s            |
| -9.63                  | 1''               | 1           | d            |
| -10.21                 | 5'                | 1           | d            |
Scheme 1

Hydrolysis

(I) Cyclic-CoA

(II) CoA

(III) Iso-CoA
Scheme 2

CoA Disulfide

NaHCO₃ pH 8.5
24h. at 37 °C

100% Yield By HPLC

EDC pH < 6.0
2 h. at 25°C

Cyclic-CoA Disulfide

1.) 15 mM β-cyclodextrin
   3M KCl, NaHCO₃ pH 9.5
2.) TCEP pH 9.5

83% Yield
By HPLC

Iso-CoA

HS-R

100% Yield By HPLC

CoA

HS-R

CoAS-S-R

R:

2

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Figure 1

A  Commercial CoA Sample

HPLC-MS

CoA  11 min.

HPLC-MS/MS

CoA Isomer  13.8 min.

HPLC-Purified Coenzyme A

CoA:  \( R_2 = H \)  \( R_3 = \text{PO}_3\text{H}_2 \)

CoA Isomer:  \( R_2 = \text{PO}_3\text{H}_2 \)  \( R_3 = H \)

B  HPLC-Purified Coenzyme A

Exact Mass Spectrum  \( (M+H)^+ = 768.1264 \)

MS/MS Precursor Ion  \( m/z 768 \)

HPLC-Purified Coenzyme A Isomer

Exact Mass Spectrum  \( (M+H)^+ = 768.1264 \)

MS/MS Precursor Ion  \( m/z 768 \)
Figure 2

A  Coenzyme A  (Earlier-eluting isomer)
   1'
   2',3'
   4'
   5'
   3''
   5''
   1''
   8''
   9''

B  Iso-Coenzyme A  (Later-eluting isomer)
   1'
   2'
   3'
   4'
   5'
   1''
   3''
   5''
   8''
   9''

ppm
Figure 3

$^1$H$^1$H Cosy of Iso-Coenzyme A$_{9''}$

Anomeric Proton

1' 2' 3' 4' 5'

H$_2$O

3'' 1'' 5'' 8'' 9''

Acetate

6''
Figure 4

$\textbf{H} - ^{31}\textbf{P} \text{ HMQC of Iso-Coenzyme A}$

The monophosphate correlates to the 2' proton.

5' phosphate
1'' phosphate

2' monophosphate
Figure 5
Figure 6

A  β-ketothiolase &
Acetoacetyl-CoA reductase

B  β-ketothiolase &
Acetoacetyl-CoA reductase
& PHB Synthase

**Figure 6**

A  β-ketothiolase &
Acetoacetyl-CoA reductase

B  β-ketothiolase &
Acetoacetyl-CoA reductase
& PHB Synthase

![Graphs showing changes in concentrations of AcCoA, Iso-AcCoA, CoA, Iso-CoA, HBCoA, and Iso-HBCoA over time.](http://www.jbc.org/Downloaded from)
Figure 7

Acid Catalyzed Synthesis of Iso-CoA

Percent of Total (CoA + Iso-CoA)

Time (Hours)

CoA

Iso-CoA
Supplementary Information

Iso-Coenzyme A

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Running Title: Iso-Coenzyme A
Commercial AcCoA

HPLC-MS

| AcCoA XIC m/z 810 | AcCoA 810 → 303 |
|-----------------|-----------------|
| 34.8 min.       | AcCoA Isomer 45.7 min. |

HPLC-MS/MS

| AcCoA | AcCoA Isomer |
|-------|--------------|
| 810 → 303 | 810 → 428 |

Acetyl-CoA: \( R_2 = H \) \( R_3 = PO_3H_2 \)
Acetyl-CoA Isomer: \( R_2 = PO_3H_2 \) \( R_3 = H \)
Commercial AcAcCoA Sample

HPLC-MS

AcAcCoA
36.4 min.

XIC m/z 852

AcAcCoA Isomer
50.9 min.

HPLC-MS/MS

852 → 136

852 → 243

852 → 261

852 → 345

852 → 428

Acetoacetyl-CoA: R₂ = H  R₃ = PO₃H₂
Acetoacetyl-CoA Isomer: R₂ = PO₃H₂  R₃ = H
Commercial HBCoA Sample

**HPLC-MS**

- HBCoA 48.1 min.
- XIC m/z 854

**HPLC-MS/MS**

- 854 → 136
- 854 → 245
- 854 → 347 HBCoA Isomer
- 854 → 428

β-hydroxybutyryl-CoA: \( R_2 = H \), \( R_1 = \text{PO}_3H_2 \)

β-hydroxybutyryl-CoA Isomer: \( R_2 = \text{PO}_3H_2 \), \( R_1 = H \)
Supplementary Figure Legends

Figure 1S: HPLC-MS and HPLC-MS/MS Analyses of Commercial Acetyl-CoA

**Samples.** HPLC-MS of commercial acetyl-CoA exhibits two distinct peaks with identical (M+H)^+ ions, m/z 810. The earlier-eluting peak is the authentic acetyl-CoA, and the later-eluting peak is acetyl-iso-CoA, which is consistent with the extensive MS and NMR analyses performed on CoA and iso-CoA. HPLC-MS/MS experiments exhibited identical product ions at the m/z’s 810→303 and m/z 810→428 transitions. The proposed fragmentation patterns are shown below.

Figure 2S: HPLC-MS and HPLC-MS/MS Analyses of Commercial Acetoacetyl-CoA Samples.

HPLC-MS of commercial acetoacetyl-CoA exhibited two distinct peaks with identical (M+H)^+ ions, m/z 852. The earlier-eluting peak is the authentic acetoacetyl-CoA, and the later-eluting peak is acetoacetyl-iso-CoA. HPLC-MS/MS experiments exhibited identical product ions at the m/z’s 852→136, 852→243, 852→261, 852→345, 852→428 transitions. The proposed fragmentation patterns are shown below.

Figure 3S: HPLC-MS and HPLC-MS/MS Analyses of Commercial β-hydroxybutyryl-CoA Samples.

HPLC-MS of commercial β-hydroxybutyryl-CoA exhibited two distinct peaks with identical (M+H)^+ ions, m/z 854. The earlier-eluting peak is the authentic β-hydroxybutyryl-CoA, and the later-eluting peak is β-hydroxybutyryl-iso-CoA. HPLC-MS/MS experiments exhibited identical product ions at the m/z’s 854→136, 854→245, 854→347, and 854→428 transitions. The proposed fragmentation patterns are shown below.
Iso-Coenzyme A
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