Synthesis of Crotonyl-OxyCoA: A Mechanistic Probe of the Reaction Catalyzed by Enoyl-CoA Hydratase

Miao Dai,[†] Yuguo Feng,[†] and Peter J. Tonge*[,†,§]

Department of Chemistry and Graduate Programs in Biophysics, Molecular and Cellular Biochemistry Molecular and Cellular Pharmacology, and Molecular Genetics and Microbiology, SUNY at Stony Brook Stony Brook, New York 11794-3400

Received September 18, 2000

A wide variety of reactions are catalyzed by enzymes that utilize coenzyme A-based substrates.[1,2] The reactions may be subdivided based on whether the acyl-CoA thioester bond is broken or formed during the reaction or whether chemical transformations are confined to the acyl portion of the molecule. The ability to probe the mechanism of CoA-dependent enzymes has been greatly facilitated by the ability to synthesize acyl-CoA analogues.[3] Here we present a method that has enabled us to synthesize a CoA oxyester for the first time. In this analogue the acyl-CoA thioester sulfur atom has been replaced by oxygen. Using this method crotonyl-oxyCoA has been synthesized and its reaction with enoyl-CoA hydratase has been studied.

The single-atom substitution of an oxygen for a sulfur is expected to influence the chemical properties of acyl-CoA esters in a number of ways, while only having a minimal effect on the geometry of the ligand.[4] First, the α-protons of the oxyester analogue will be more basic than those of the corresponding thioester. Richard and co-workers have shown that the α-protons of ethyl acetate (pK_a 25.6) are 40 000-fold more basic than the α-protons of ethyl thiolacetate (pK_a 21).[5,6] Thus, the oxyester should be a sensitive probe of the role of α-proton acidity in an enzyme reaction. Second, the carbonyl stretching frequency of oxyesters is intrinsically higher in wavenumber than that of the corresponding thioester. For example, in methyl acetate $\nu_{C=O}$ is 1740 cm^{-1} while $\nu_{C=O}$ is 1692 cm^{-1} for methyl thiolacetate.[7] The shift in frequency is expected to facilitate the direct observation of $\nu_{C=O}$ when the acyl-oxyCoA ligands are bound to enzymes, especially in compounds such as α,β-unsaturated fatty acids where the carbonyl is conjugated to an ethylenic double bond. Third, while the alkaline hydrolysis rate constants for oxygen and thioesters are comparable,[8,9] the free energy of hydrolysis of thioesters is around 2.5 kcal/mol greater than that for the corresponding oxyesters.[10,11] Consequently, the increased thermodynamic stability of oxyesters relative to thioesters and, for example, the fact that oxyesters are much less reactive toward nitrogen nucleophiles than thioesters[12] is expected to facilitate the investigation of enzyme reactions that involve cleavage of the acyl-CoA ester bond and assist in the generation of stable enzyme−substrate complexes for spectroscopic analysis. Consequently the acyl-oxyCoAs should be valuable mechanistic probes of enzymes that utilize CoA substrates.

Initial efforts to synthesize acyl-oxyCoA esters were based on the method described by Drueckhammer and co-workers.[3,13,14] This procedure utilizes a common CoA intermediate in which the pantetheine amide bond closest to the CoA thiol group has been replaced by a thioester. Subsequent aminolysis of the thioester reforms the pantetheine amide and results in a CoA analogue whose structure is derived from that of the amine nucleophile. To introduce an oxyester functionality using this method, we consequently required compounds containing both an amine and an oxyester. However, we observed that an intramolecular N to O rearrangement of the required amino acyl-oxyester fragment competed efficiently with the aminolysis of the CoA intermediate. Subsequently, we turned our attention to the synthesis of acyl-oxypantetheines, based on the hypothesis that the enzymes responsible for the synthesis of CoA from pantetheine might accept a variety of substituted pantetheine derivatives.

Crotonyl-oxypantetheine **3**, the acyl-oxypantetheine required for the synthesis of crotonyl-oxyCoA, was obtained from D-pantothenic acid as shown in Scheme 1.[15] Following protection of the pantothenate hydroxyl groups with triethylsilyl chloride, pantothenic acid was coupled with ethanolamine using carbonyl diimidazole to give the protected oxypantetheine **2** in 40% yield. The oxypantetheine **2** was then acylated with crotonic acid using DCC and deprotected to give crotonyl-oxypantetheine **3** in 53% yield.

Crotonyl-oxypantetheine **3** was subsequently converted into crotonyl-oxyCoA **6** with three enzymatic steps (Scheme 1).[15] Initially, pantothenate kinase (pantK) was used to phosphorylate crotonyl-oxypantetheine **3** with ATP to give crotonyl-oxyphosphopantetheine **4**. The addition of pyruvate kinase and phosphoenolpyruvate to the reaction mixture was found to increase the yield of the phosphorylated product. The progress of the reaction was monitored by using lactate dehydrogenase to reduce the pyruvate that was formed to lactate, with the concomitant oxidation of NADH. By using the coupled assay, crotonyl-oxypantetheine was found to be an excellent substrate for pantK. Phosphorylation occurred quantitatively and, following purification, the crotonyl-oxyphosphopantetheine was coupled with ATP by using phosphopantetheine adenylyltransferase (PPAT) to give crotonyl-oxydephosphoCoA **5**. Pyrophosphatase was added to the coupling reaction to hydrolyze the inorganic pyrophosphate that was formed. Finally, the crotonyl-oxydephosphoCoA **5** was converted into crotonyl-oxyCoA **6** by using 3′-dephosphoCoA kinase (dpCoAK). Each enzymatic step proceeded quantitatively to product and the overall yield of the enzymatic portion of the synthesis following HPLC purification of the final product was 62%.

The ability of enoyl-CoA hydratase to hydrate crotonyl-oxyCoA was subsequently examined. Enoyl-CoA hydratase catalyzes the syn hydration of α,β-unsaturated fatty acyl-CoAs to the corresponding 3(*S*)-hydroxyacyl-CoAs (Scheme 2).[16] For crotonyl-CoA

* To whom correspondence should be addressed. Telephone: (631) 632 7907. Fax: (631) 632 7907. E-mail: peter.tonge@sunysb.edu.

[†] Department of Chemistry.

[§] Graduate Programs in Biophysics, Molecular and Cellular Biochemistry, Molecular and Cellular Pharmacology and Molecular Genetics and Microbiology.

(1) McCormick, D. B.; Wright, L. D. *Methods Enzymology*; Academic Press: New York, 1970; Vol. 18A, Chapter 4.

(2) Lee, C-Y.; Chen, A. F. In *The Pyridine Nucleotide Coenzymes*; Everse, J., Anderson, B., You, K-S., Eds.; Academic Press: New York, 1982; p 189.

(3) Mishra, P. K.; Drueckhammer, D. G. *Chem. Rev.* **2000**, *100*, 3283−3310.

(4) Zacharias, D. E.; Murrayrust, P.; Preston, R. M.; Glusker, J. P. *Arch. Biochem. Biophys.* **1983**, *222*, 22−34.

(5) Amyes, T. L.; Richard, J. P. *J. Am. Chem. Soc.* **1992**, *114*, 10297−10302.

(6) Amyes, T. L.; Richard, J. P. *J. Am. Chem. Soc.* **1996**, *118*, 3129−3141.

(7) Elaasar, A. M. M.; Nash, C. P.; Ingraham, L. L. *Biochemistry* **1982**, *21*, 1972−1976.

(8) Rylander, P. N.; Tarbell, D. S. *J. Am. Chem. Soc.* **1950**, *72*, 3021−3025.

(9) Morse, B. K.; Tarbell, D. S. *J. Am. Chem. Soc.* **1952**, *74*, 416−419.

(10) Jencks, W. P.; Cordes, S.; Carriuolo, J. *J. Biol. Chem.* **1960**, *235*, 3608−3614.

(11) Bruice, T. C.; Benkovic, S. J. *Bioorganic Mechanisms*; W. A. Benjamin: New York, 1966; pp 268−294.

(12) Connors, K. A.; Bender, M. L. *J. Org. Chem.* **1961**, *26*, 2498−2504.

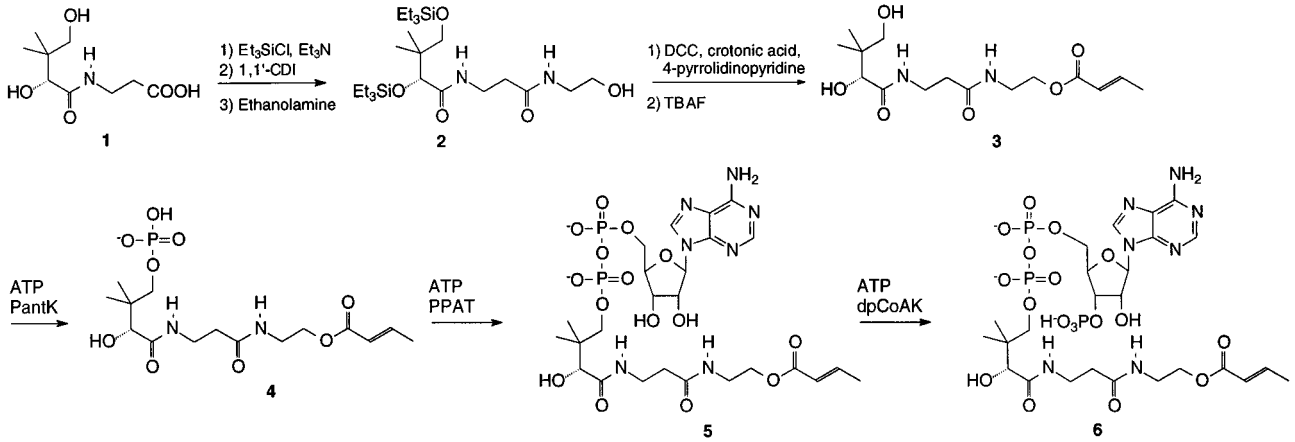
(13) Martin, D. P.; Drueckhammer, D. G. *J. Am. Chem. Soc.* **1992**, *114*, 7287−7288.

(14) Martin, D. P.; Bibart, R. T.; Drueckhammer, D. G. *J. Am. Chem. Soc.* **1994**, *116*, 4660−4668.

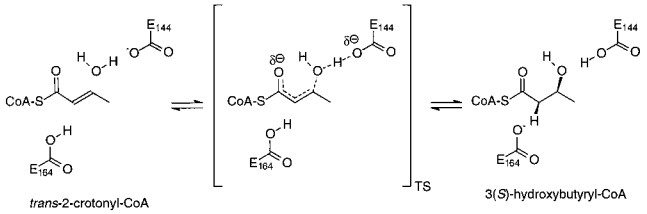
(15) Detailed procedures are given in the Supporting Information.

10.1021/ja003406k CCC: $20.00
Published on Web 12/30/2000

Scheme 1



Scheme 2



the equilibrium constant for hydration to 3(*S*)-hydroxybutyryl-CoA (K_{HB}) is 7.5 in D_2O buffer (pD 7.4).[17] Comparison of the UV−visible absorption spectrum of crotonyl-oxyCoA before and after addition of enzyme revealed that an absorbance band around 220 nm, assigned to the enoyl group, decreased substantially during the enzymatic reaction. HPLC analysis of the reaction mixture revealed that the crotonyl-oxyCoA, retention time 29.3 min, was replaced by a new product at 26.6 min. The product was isolated and shown to react quantitatively with 3(*S*)-hydroxyacyl-CoA dehydrogenase and NAD^+ in a coupled reaction with pyruvate and lactate dehydrogenase. This confirmed that the product of the reaction was 3(*S*)-hydroxybutyryl-oxyCoA. Using NMR spectroscopy, we determined that the equilibrium constant for the hydration of crotonyl-oxyCoA (K_{HB}) was 12.5. This is larger than the equilibrium constant for the hydration of crotonyl-CoA (7.5). Consequently the sulfur-to-oxygen substitution has destabilized the enoyl system and/or stabilized the hydrated product.

The hydration of crotonyl-CoA at pH 7.4 is characterized by a k_{cat} of 1280 s^{-1} and a K_m of 15 μM giving a k_{cat}/K_M of 8.5×10^7 M^{-1} s^{-1}. In contrast, the kinetic parameters for the hydration of crotonyl-oxyCoA under the same conditions were found to be a k_{cat} of 3.9 s^{-1} and a K_m of 13 μM, giving a k_{cat}/K_M of 3.0×10^5 M^{-1} s^{-1}. Thus, replacement of the crotonyl-CoA thioester linkage with an oxyester reduces k_{cat} for substrate hydration by 330-fold and k_{cat}/K_M by 280-fold. Clearly, the single-atom substitution has had a dramatic effect on reactivity. While the origins of the alteration in activity remain to be explored, the lower acidity of the α-protons of oxyesters compared to those of thioesters indicates that the thioester group is intrinsically better at stabilizing the carbanion resulting from α-proton abstraction compared to the oxyester group.[5,6,18] Consequently, since the transition state for the concerted hydration of crotonyl-CoA has some carbanionic character (Scheme 2),[19,20] the reduced reactivity of crotonyl-oxyCoA may simply reflect the reduced ability of the oxyester to stabilize negative charge accumulation in the acyl group.

In summary, we have synthesized an acyl-oxyCoA for the first time. The general applicability of this method for synthesizing further CoA analogues will depend on the ability of the CoA biosynthesis enzymes to accept different pantetheine analogues. Currently, in addition to crotonyl-oxyCoA, we have demonstrated that the CoA biosynthesis enzymes can accept pantetheines with C6 (hexadienoyl-oxypantetheine) and C12 (2-dodecenoyl-pantetheine) fatty acid substituants. The acyl-oxyCoA analogues will prove a highly valuable tool for probing the mechanism of CoA-dependent enzymes.

Acknowledgment. This work was supported by NSF Grant MCB-9604254. We thank Professor Dale Drueckhammer and Dr. Pranab Mishra for helpful discussions and for providing the pantothenate kinase and phosphopantetheine adenylyltransferase enzymes. The NMR facility at SUNY Stony Brook is supported by grants from NSF (CHE9413510) and from NIH (1S10RR554701).

Supporting Information Available: Detailed descriptions of experimental procedures (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA003406K

(16) Willadsen, P.; Eggerer, H. *Eur. J. Biochem.* **1975**, *54*, 247−252.
(17) Wu, W. J.; Feng, Y.; He, X.; Hofstein, H. S.; Raleigh, D. P.; Tonge, P. J. *J. Am. Chem. Soc.* **2000**, *122*, 3987−3994.
(18) Gerlt, J. A.; Gassman, P. G. *Biochemistry* **1993**, *32*, 11943−11952.
(19) Bahnson, B. J.; Anderson, V. E. *Biochemistry* **1989**, *28*, 4173−4181.
(20) Bahnson, B. J.; Anderson, V. E. *Biochemistry* **1991**, *30*, 5894−906.