4-Thia-*trans*-2-alkenoyl-CoA Derivatives: Properties and Enzymatic Reactions†

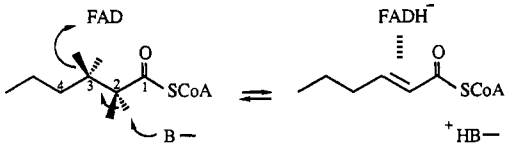
Sze-Mei Lau, Richard K. Brantley, and Colin Thorpe*

Department of Chemistry and Biochemistry, University of Delaware, Newark, Delaware 19716

Received March 30, 1989

ABSTRACT: 4-Thiaacyl-CoA analogues, in which the 4-methylene group is replaced by a thioether sulfur atom, represent new chromophoric substrates of acyl-CoA dehydrogenases and oxidase. The corresponding 4-thia-*trans*-2-enoyl-CoA products exhibit a strong new absorption band (extinction coefficient 22 mM^{-1} cm^{-1}) that is red shifted from 312 to 338 nm upon binding to the medium-chain acyl-CoA dehydrogenase. 4-Thiaoctanoyl-CoA reduces the dehydrogenase several-fold slower than octanoyl-CoA, although in turnover it is dehydrogenated 1.5-fold faster. The redox potential of 4-thia analogues is some 30 mV more negative than that of their unsubstituted counterparts. 4-Thia-*trans*-2-enoyl-CoA derivatives are slowly hydrated by enoyl-CoA hydratase (EC 4.2.1.17) to the corresponding thiohemiacetal which fragments nonenzymatically to 1 equiv each of malonylsemialdehyde-CoA and alkanethiol. This fragmentation reaction might explain the release of methanethiol during the transamination pathway of methionine degradation. 4-Oxaoctanoyl-CoA is a much poorer substrate and kinetic reductant of acyl-CoA dehydrogenase and oxidase than the 4-thia analogue. The corresponding enoyl-CoA product is also fragmented by the hydratase, yielding butanol and malonylsemialdehyde-CoA. Thus, 4-heterosubstituted acyl-CoA derivatives provide new tools for the study of β-oxidation enzymes.

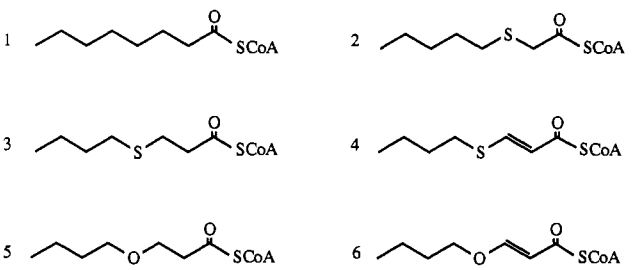
Recently we have begun studies using heteroatom-substituted acyl-CoA[1] analogues as probes for β-oxidation enzymes (Thorpe, 1987; Lau et al., 1988b). The initial focus of this work was the medium-chain acyl-CoA dehydrogenase from pig kidney (Thorpe et al., 1979). The reductive half-reaction of this flavoprotein involves removal of the pro-R-α hydrogen as a proton, with the concerted elimination of the pro-R-β hydrogen as a hydride (Murfin, 1974; Ghisla et al., 1984; Pohl et al., 1986; Schopfer et al., 1988):



Replacement of the C-3 methylene group with an oxa or thia substituent (Chart I; e.g., compound 2) prevents effective transfer of reducing equivalents to the flavin so that these derivatives are not substrates of the enzyme (Lau et al., 1988b). Proton abstraction does, however, occur, leading to enzyme-bound enolates that participate in charge-transfer complexes with the adjacent oxidized flavin. Some of these species show intense long-wavelength absorbance (e.g., 9000 mM^{-1} cm^{-1} at 800 nm), making them useful spectral probes of the acyl-CoA dehydrogenases (Lau et al., 1988b).

This paper describes the synthesis and characterization of the products of the enzymatic oxidation of 4-thia- and 4-oxaoctanoyl-CoA and related derivatives (e.g., Chart I; compounds 3 and 5 to compounds 4 and 6, respectively). The 4-thia analogues prove particularly interesting chromophoric derivatives of normal *trans*-2-enoyl thioesters. Further, both oxa- and thiaenoyl-CoA derivatives are subject to a very interesting fragmentation reaction following hydration with enoyl-CoA hydratase (crotonase, EC 4.2.1.17). In addition to providing new tools for the study of β-oxidation, this reaction might explain the release of methanethiol during the transamination pathway of methionine metabolism.

Chart I: Octanoyl-CoA Analogues Described in the Text[a]



[a] Compound **1**, octanoyl-CoA; compound **2**, 3-thiaoctanoyl-CoA; compound **3**, 4-thiaoctanoyl-CoA; compound **4**, 4-thia-*trans*-2-octenoyl-CoA; compound **5**, 4-oxaoctanoyl-CoA; compound **6**, 4-oxa-*trans*-2-octenoyl-CoA.

Materials and Methods

Materials. Medium-chain acyl-CoA dehydrogenase was isolated from pig kidneys as described previously (Lau et al., 1988b). Acyl-CoA oxidase was purified from *Candida tropicalis* as in Jiang and Thorpe (1983). The short-chain acyl-CoA dehydrogenase was isolated from pig liver by a modification of the method of Shaw and Engel (1984) and was a gift from Nicole Nataro. The concentrations of these flavoproteins is reported in terms of bound flavin by using extinction coefficients of 15.4 (Thorpe et al., 1979) and 14.9 mM^{-1} cm^{-1} (Shaw & Engel, 1984) for medium- and short-chain acyl-CoA dehydrogenase, respectively. CoASH (lithium salt) and crotonyl- and octanoyl-CoA were obtained from Pharmacia. D-Pantethine, NAD^+, crotonase, 3-OH-acyl-CoA dehydrogenase, and horse liver alcohol dehydrogenase were from Sigma. 3-Methylthiopropionic acid (4-thiapentanoic acid) was from CTC Organics. 4-Thiaoctanoic, 4-thiadodecanoic, 3-(phenylthio)propionic, and 4-oxaoctanoic acids were

† This work was supported in part by a grant from the U.S. Public Health Service (GM 26643)

[1] Abbreviations: CoA, coenzyme A; HPLC, high-performance liquid chromatography.

0006-2960/89/0428-8255$01.50/0

synthesized and characterized as described earlier (Lau et al., 1988b). Free acids larger than octanoic were purified by recrystallization from hexane or methanol. Acids were converted to their corresponding CoA thioesters by the mixed-anhydride procedure (Bernert & Sprecher, 1977; Powell et al., 1987) and purified by HPLC as described by Lau et al. (1988b). All CoA thioesters were characterized by proton NMR recorded in D_2O by use of a Bruker 250-mHz spectrometer equipped with an Aspect 3000 computer system.

General. Unless otherwise stated, all buffers contained 0.3 mM EDTA, and enzyme incubations were performed at 25 °C. Assays of the medium-chain acyl-CoA dehydrogenase were as described previously (Thorpe, 1981); the short-chain enzyme was assayed essentially as described by Shaw and Engel (1984). Anaerobic experiments were as described previously (Gorelick et al., 1982). Spectrophotometric titrations were recorded on a Cary 219 instrument interfaced to a microcomputer (Powell et al., 1987). Ligand binding was assessed by a nonlinear regression analysis program (F-curve II) written by Dr. Joseph Noggle of this department. Rapid reaction experiments utilized a Kinetic Instruments Stopped Flow equipped with a 2-cm absorbance flow cell with peripherals and software for data acquisition and fitting from Online Instruments Systems.

Synthesis of (4-Thiaoctanoyl)pantetheine. D-Pantetheine (227 mg) was added to 4 mL of water and stirred for 30 min with 20 mg of sodium borohydride, and then excess reductant was quenched with 0.5 mL of acetone. The mixed anhydride of 4-thiaoctanoic acid was prepared separately by using 162 mg of the acid in 14 mL of dry tetrahydrofuran, adding 140 μL of triethylamine and then 95 μL ethylchloroformate. Both solutions were cooled to 0 °C, mixed, and stirred for 10 min. The upper phase was collected, washed with 5 mL of 10% (w/v) potassium carbonate, and dried over anhydrous sodium sulfate. The solvent was evaporated and the product redissolved in 3 mL of 50% (v/v) ethanol. The thioester was purified by HPLC on a semipreparative octadecylsilica reverse-phase column at 3 mL/min using a methanol/water system (0% methanol, 5 min; followed by a linear gradient between 50 and 100% methanol lasting 15 min). Peaks were pooled according to their absorbance at 230 nm and checked for the presence of thioesters by using the nitroprusside test (Stadtman, 1957). The thioester-positive product was recovered by evaporation of the solvent and characterized by NMR in deuterochloroform.

Preparation of 4-Thia-trans-2-octenoyl-CoA. Acyl-CoA oxidase (20 μL of 100 μM enzyme) was added to 11 mL of 0.73 mM 4-thiaoctanoyl-CoA in 50 mM phosphate buffer, pH 7.6, in a 250-mL beaker. The solution was gently stirred at room temperature, and small samples were withdrawn to monitor the progress of the reaction at 312 nm (see text). The mixture was ultrafiltered after 2 h, yielding a filtrate that was concentrated to about 1 mL by rotary evaporation, and subsequently purified by HPLC on a semipreparative reverse-phase column using a gradient of methanol in 50 mM potassium phosphate, pH 5.3 (Corkey et al., 1981; 30% methanol held for 5 min and then increased to 70% over 15 min). The enoyl-CoA product eluted at 20.8 min, about 1.5 min after 4-thiaoctanoyl-CoA (see Results). The product was desalted by gel filtration on Bio-Gel P-2 and stored as a lyophilized white powder at −20 °C. No significant decomposition was detected over several months under these conditions. Solutions of the 4-thiaenoyl-CoA derivative in 50 mM buffer at room temperature proved to be rather stable monitored by UV spectra: the decline in absorbance at 312 nm over 3 days was insignificant in phosphate, pH 5.6, and about 5% in both phosphate, pH 7.6, and Tris, pH 8.8.

Proton NMR in D_2O shows the expected resonances for an enoyl-CoA thioester. In particular, single-proton doublets at 7.8 and 6.2 ppm (overlapping the 1′-H resonance of the ribose moiety) with coupling constants of about 15 Hz are consistent with C-3 and C-2 resonances of a 4-thia-*trans*-2-enoyl derivative, respectively (Silverstein et al., 1981). The methylene protons adjacent to the vinyl thioether showed a triplet at 2.9 ppm.

The extinction coefficient of 4-thiaoctenoyl-CoA was determined to be 22 mM^{-1} cm^{-1} at 312 nm by reductive cleavage of the thioester. 4-Thia-*trans*-2-octenoyl-CoA (about 40 μM in 0.72 mL of 50 mM phosphate buffer, pH 7.6) was treated with 20 mg of sodium borohydride for 1 h at room temperature. Excess borohydride was discharged with 140 μL of 4 M HCl followed by neutralization of the mixture with 20 μL of 4 M KOH. The combined solution was brought to 0.5 mM DTNB and the absorbance recorded at 412 nm (Degani & Patchornik, 1971) versus blanks without the thioester. Simple base-catalyzed hydrolysis cannot be used for the quantitation of enoyl thioesters because of Michael addition of the liberated CoASH across the double bond (Stadman, 1957; Lau & Thorpe, 1988a).

Preparation of (4-Thiaoctenoyl)pantetheine. (4-Thiaoctanoyl)pantetheine (40 μM in 720 μL of 50 mM phosphate buffer, pH 7.6) was incubated overnight with 1 μM acyl-CoA oxidase following the reaction by the appearance of a peak at 312 nm (see text). The product was extracted by using two aliquots of 2 mL of chloroform, dried over anhydrous sodium sulfate, recovered by evaporation of the solvent, and purified by HPLC using methanol/water (see above).

Preparation of 4-Oxaoctenoyl-CoA. 4-Oxaoctanoyl-CoA (110 μM) was oxidized by 0.9 μM acyl-CoA dehydrogenase with 345 μM ferricenium hexafluorophosphate (T. Lehman, and C. Thorpe, unpublished observations) as terminal acceptor in a total volume of 1.46 mL of 50 mM phosphate buffer, pH 7.6. The reaction was followed by the decrease in absorbance of the ferricinium ion at 617 nm upon reduction to ferrocene. The reaction had stopped after 30 min, and the mixture was adjusted to pH 5 with HCl. The enoyl-CoA product was purified by HPLC (4-oxaoctanoyl- and the corresponding *trans*-2-enoyl-CoA derivative elute at 15.8 and 18.7 min, respectively, when the gradient described earlier is used).

Synthesis of Malonylsemialdehyde-CoA. Crotonase (20 μg) was added to 1 mL of 36 μM propynoyl-CoA (Freund et al., 1985; Thorpe, 1986) in 50 mM phosphate buffer, pH 7.6. The reaction was followed at 300 nm to completion at room temperature (about 15 min). The solution was centrifuged by using Amicon microconcentrators (Centricon) to remove crotonase and the filtrate used immediately without further purification. Malonylsemialdehyde-CoA shows an apparent extinction coefficient at 299 nm of 14.5 mM^{-1} cm^{-1} at pH 7.6 and of 21.3 mM^{-1} cm^{-1} at pH 11 (upon the addition of KOH; Thorpe, 1986).

Assays Involving Enoyl-CoA Hydratase. The standard assay contained a total volume of 700 μL of 100 mM potassium phosphate buffer, pH 7.4, 25 °C, with 0.3 mM EDTA, 70 μg of serum albumin, and 10–170 μM 4-thia-*trans*-2-pentenoyl-CoA or crotonyl-CoA. Reactions were started by the addition of crotonase and were followed by the decrease in absorbance at 320 and 280 nm for thia and normal analogues, respectively (see text).

Thiol release from 60 μM 4-thia-*trans*-2-octenoyl-CoA was estimated in 820 μL of 50 mM phosphate buffer, pH 7.6,

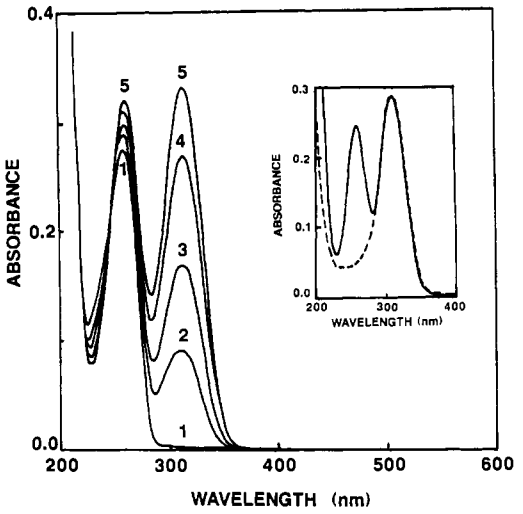


FIGURE 1: Oxidation of 4-thiaoctanoyl-CoA to 4-thia-*trans*-2-octenoyl-CoA by acyl-CoA oxidase from *C. tropicalis*. Acyl-CoA oxidase was diluted to 14 nM in 17 μM 4-thiaoctanoyl-CoA in 50 mM phosphate buffer, pH 7.6, 25 °C (curve 1). Spectra 2–5 were recorded 2, 4, 16, and 70 min after the addition of enzyme. The inclusion of 1 μg of bovine liver catalase had no effect on the course of these spectral changes (data not shown). The inset compares the spectrum of 4-thia-*trans*-2-octenoyl-CoA (—) and that of the corresponding pantetheine analogue (---) after purification by HPLC (see Materials and Methods).

containing 0.5 mM DTNB. The reaction was initiated by the addition of 120 μg of crotonase and the absorbance at 412 nm (Degani & Patchornik, 1971) measured versus a blank minus 4-thia analogue. No reaction was observed in the absence of crotonase.

Release of butanol from 4-oxaoctenoyl-CoA was measured in 800 μL of 100 mM Tris buffer, pH 8.8, 25 °C, in the presence of 0.3 mM EDTA, 5 mM NAD^+, and 80 μg of alcohol dehydrogenase. The background absorbance change at 340 nm was recorded and the reaction initiated by the addition of 80 μg of crotonase.

Results

Synthesis and Characterization of 4-Thia-trans-2-octenoyl-CoA. Figure 1 shows the spectral changes observed on incubation of the saturated analogue 4-thiaoctanoyl-CoA (Lau et al., 1988b) with acyl-CoA oxidase in aerobic buffer, pH 7.6. These oxidations can be conveniently followed spectrophotometrically with acyl-CoA oxidase by using molecular oxygen as the terminal oxidant. In addition to the expected absorption of the adenine moiety at 260 nm, a prominent new band appears at 312 nm (curves 2–5, Figure 1) due to the formation of the corresponding *trans*-2-enoyl derivative (see below). The changes shown in Figure 1 suggest a smooth conversion of substrate into product without the accumulation of spectrally detectable intermediates. Similarly, sampling the reaction mixture by HPLC on a reverse-phase column (see Materials and Methods) showed the disappearance of the substrate peak at 19.3 min, with the appearance of a single peak eluting at 20.8 min. It should be noted that this order of elution is unexpected, since unsaturated CoA thioesters almost invariably elute more rapidly than their saturated counterparts on reverse-phase columns (e.g., octenoyl- and octanoyl-CoA; Lau & Thorpe, 1988a). The same unusual chromatographic procedure is also seen with 4-thia-*trans*-2-pentenoyl-CoA and with 4-oxa-*trans*-2-octenoyl-CoA to be described later.

The proton NMR spectrum of the product obtained from large-scale incubations (see Materials and Methods) of 4-thiaoctanoyl-CoA with acyl-CoA oxidase is consistent with the expected formation of a *trans*-2-enoyl-CoA product (Kawaguchi et al., 1980). The peak position in the UV spectrum is also consistent with that expected for a 4-thia-substituted enone system (Silverstein et al., 1981). In addition, the magnitude of the extinction coefficient for this 312-nm band (22 mM^{-1} cm^{-1}; see Materials and Methods) is also suggestive of a trans configuration about the double bond (Silverstein et al., 1981). Finally, enzymatic hydration of the unsaturated 4-thia derivative yields the expected fragmentation products (see later).

Consistent with the results obtained with the oxidase, incubation of 4-thiaoctanoyl-CoA with the medium-chain acyl-CoA dehydrogenase using ferricenium hexafluorophosphate as terminal oxidant (Lehman and Thorpe, unpublished observations) yields the increase at 312 nm expected for the accumulation of 4-thiaenoyl product.

Preparation of 4-Thia-trans-2-pentenoyl-CoA, 4-Thia-trans-2-dodecenoyl-CoA, and (4-Thia-trans-2-octenoyl)pantetheine Derivatives. Analogues with acyl chains both shorter and longer than 4-thia-*trans*-2-octenoyl-CoA could be prepared easily by acyl-CoA oxidase treatment. Pentenoyl- and dodecenoyl-CoA analogues had essentially the same electronic spectra as that shown by the *trans*-2-octenoyl-CoA derivative (data not shown). In addition, the phenylthio analogue *trans*-3-(phenylthio)acryloyl-CoA was synthesized by oxidation of 3-(phenylthio)propionyl-CoA and also showed a peak at 312 nm (see Materials and Methods).

The pantetheine analogue of 4-thia-*trans*-2-octenoyl-CoA was also prepared by oxidation using acyl-CoA oxidase (see Materials and Methods). The spectrum of this derivative is shown in the inset to Figure 1. As expected, (4-thiaoctenoyl)pantetheine shows a dominant peak at 312 nm without the second peak at 260 nm associated with the adenine chromophore.

Redox Potential of 4-Thiaacyl-/4-Thiaenoyl-CoA Couple. Preliminary work showed that 4-thiaoctanoyl-CoA was an effective reductant of the medium-chain acyl-CoA dehydrogenase (Lau et al., 1988b). In particular, the bleaching of the flavin chromophore and the appearance of a long-wavelength band were similar to those seen with octanoyl-CoA. It was therefore of interest to compare the redox potential of normal and 4-thia substrates.

Medium-chain acyl-CoA dehydrogenase catalyzes a slow transhydrogenation reaction (Murfin, 1974; McFarland et al., 1982) involving the couples shown:

4-thiapentanoyl-CoA + crotonyl-CoA $\rightleftharpoons$
4-thiapentenoyl-CoA + butyryl-CoA

This reaction is too slow to be useful as an assay of the dehydrogenase (turnover number about 3/min; data not shown), but it does allows determination of the equilibrium constant of this reaction. Catalytic levels of the dehydrogenase were used under anaerobic conditions (see Materials and Methods) to avoid the slow oxidase activity of the enzyme commonly seen with shorter substrates (Steyn-Parvé & Beinert, 1958; Thorpe et al., 1979; McFarland et al., 1982). Equilibration from left to right is accompanied by the appearance of a band at 312 nm due to the 4-thia unsaturated product, allowing the concentrations of each species to be calculated (Table I). Similar values for the equilibrium constant were obtained by starting from either side of the equation (with an average value of 13.5; Table I).

The redox potential for the 4-thiapentanoyl-/-pentenoyl-CoA couple can then be estimated as −0.046 V versus the standard hydrogen electrode by combining this equilibrium constant with a recently redetermined value of −0.013 V for

Table I: Equilibration in the Transhydrogenation Reaction Catalyzed by Medium-Chain Acyl-CoA Dehydrogenase[a]

expt	crotonyl-CoA (μM)	4-thia-alkanoyl-CoA (μM)	butyryl-CoA (μM)	4-thia-*trans*-2-enoyl-CoA (μM)	equi-librium constant
A					
initial	36.5	38.6	0	0	
final	7.2	9.3	29.3	29.3	12.8
B					
initial	0	0	33.1	42.6	
final	7.9	7.9	25.3	34.7	14.1
C					
initial	17.1	19.1	0	0	
final	2.7	4.7	14.3	14.3	16.1
D					
initial	0	0	21.5	18.1	
final	4.5	4.5	17	13.6	11.4

[a] The initial concentrations of reagents noted in experiments A–D were deoxygenated in an anaerobic cuvette in a total volume of 0.9 mL of 50 mM phosphate buffer, pH 7.6, 25 °C. 4-Thiapentanoyl-CoA was used in experiments A and B and the corresponding octanoyl analogue in experiments C and D. After an initial spectrum was recorded, medium-chain enzyme was added from a sidearm to a final concentration of 0.87 μM, and the absorbance of the solution at 312 nm was followed over the 2–3 h required for equilibration. The average equilibrium constant is 13.6 ± 2.5.

Table II: Rate Constants for the Reduction of Medium-Chain Acyl-CoA Dehydrogenase with Octanoyl-CoA and 4-Thiaoctanoyl-CoA[a]

substrate	rate constants (s^{-1}) 340 nm	446 nm	570 nm
octanoyl-CoA	ND[b]	335	437
		59	77
		5	4
4-thiaoctanoyl-CoA	49	48	45
	8[c]	10[c]	6[c]

[a] Conditions as in Figure 2. [b] Not determined. [c] The slower phase observed with 4-thiaoctanoyl-CoA accounts for 10–15% of the total absorbance change at these wavelengths.

the butyryl-/crotonyl-CoA couple (Stankovich & Soltysik, 1987). Similarly, the redox potential for the 4-thiaoctanoyl derivatives was estimated as −0.047 mV (Table I). Equilibration in this system was somewhat slower and presumably limited by the dissociation of the longer enoyl-CoA analogues from the reduced dehydrogenase.

Kinetics of the Reduction of Medium-Chain Acyl-CoA Dehydrogenase by 4-Thiaoctanoyl-CoA. 4-Thiaoctanoyl-CoA is an excellent substrate of the medium-chain acyl-CoA dehydrogenase being oxidized approximately 1.5-fold more rapidly than octanoyl-CoA in the standard dye assay using phenazine methosulfate and dichlorophenolindophenol (Lau et al., 1988b). However, turnover of octanoyl-CoA in this assay is limited by the oxidative half-reaction and not by the rapid reduction of the enzyme by substrate (Murfin, 1974; Hall et al., 1979; Gorelick et al., 1985). Figure 2 compares the reduction of the medium-chain acyl-CoA dehyrogenase by octanoyl-CoA and 4-thiaoctanoyl-CoA in the stopped-flow spectrophotometer at 1 °C. Clearly, 4-thiaoctanoyl-CoA is a significantly slower reductant of the medium-chain enzyme following either reduction of the flavin (446 nm) or accumulation of the charge-transfer complex at 570 nm. A more quantitative comparison is difficult because the kinetic traces for octanoyl-CoA are apparently at least triphasic, whereas those for the 4-thia analogue are biphasic, and the question arises as to which phases to compare. Table II lists the apparent rate constants from best fits (see Materials and Methods) to the curves shown in Figure 2. Note that 340 nm is close to an isosbestic point for the reduction of the dehydrogenase by the normal substrate octanoyl-CoA (Thorpe et al., 1979), whereas with 4-thiaoctanoyl-CoA a marked increase is seen (Figure 2A). This feature is largely due to the accumulation of bound 4-thia-*trans*-2-octenoyl-CoA (see below), and thus we can independently monitor product formation and compare it with the reduction of the flavin and with the formation of the purple charge-transfer species (panels B and C, respectively, of Figure 2). Table II shows that the apparent rates at 340, 446, and 570 nm for the fast phase using 4-thiaoctanoyl-CoA are very similar to one another (about 47 s^{-1} at 1 °C). These data provide further evidence that the purple species formed on the addition of good substrates to the medium-chain acyl-CoA dehydrogenase represent a reduced flavin-product complex (see Discussion).

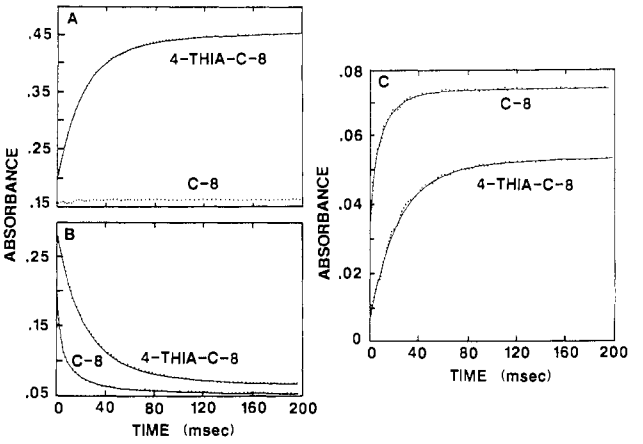


FIGURE 2: Comparison of the rates of reduction of the medium-chain acyl-CoA dehydrogenase using octanoyl- and 4-thiaoctanoyl-CoA. Solutions of the dehydrogenase (20 μM) and substrate (84 μM) in phosphate buffer, pH 7.6, 1 °C, were mixed in a stopped-flow spectrophotometer, and the absorbance was recorded at 340, 446, and 570 nm (dotted lines, panels A–C, respectively). The solid lines represent best fits to the data assuming three or two simultaneous exponential processes for octanoyl-CoA and 4-thiaoctanoyl-CoA, respectively (see also Table II).

Interaction of 4-Thia-trans-2-octenoyl-CoA with Oxidized Pig Kidney Medium-Chain Acyl-CoA Dehydrogenase. Reduction of the acyl-CoA dehydrogenase by 4-thiaoctanoyl-CoA leads to the appearance of a band at 338 nm (see above) that is not seen with octanoyl-CoA. However, 4-thia-*trans*-2-octenoyl-CoA absorbs at 312 nm in solution (Figure 1), and it was thus of interest to examine whether such a large (about 25 nm) red shift would also be observed upon binding to the oxidized dehydrogenase. Figure 3 shows that 4-thia-*trans*-2-octenoyl-CoA and the normal enoyl analogue, *trans*-2-octenoyl-CoA, effect very similar changes to the flavin absorbance envelope above 400 nm (see inset; Powell et al., 1987). The marked difference seen at lower wavelengths, notably a peak at 338 nm with the 4-thia derivative, is attributed to the contribution of the bound chromophoric ligand. It should be noted that the 4-thia analogue binds sufficiently tightly to the enzyme (K_d = 470 nM, stoichiometry 0.87/molecule ligand/FAD; data not shown) to ensure a small contribution of free ligand in these difference spectra. As expected, the addition of excess 4-thiaenoyl-CoA leads to the appearance of increased 312-nm absorbance (data not shown). Thus, the red shift encountered with the reduced enzyme–4-thia-*trans*-2-enoyl-CoA complex (see above) is also observed upon binding of the enoyl-CoA species to the oxidized enzyme.

Hydration of 4-Thia-trans-2-octenoyl-CoA by Crotonase: Identification of the Products. This section will show that treatment of 4-thia-*trans*-2-octenoyl-CoA with an enoyl-CoA

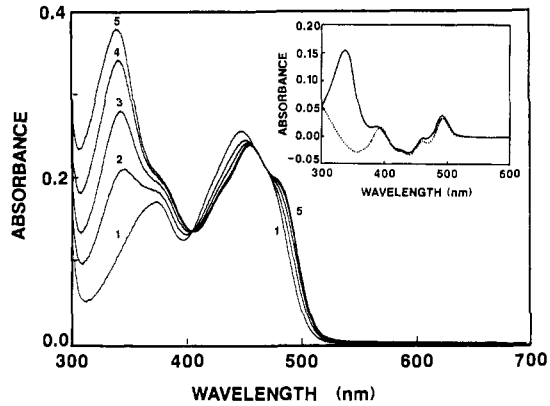


FIGURE 3: Titration of medium-chain acyl-CoA dehydrogenase with 4-thia-*trans*-2-octenoyl-CoA. The dehydrogenase (16.6 μM enzyme flavin in 50 mM phosphate buffer, pH 7.6, 25 °C,; curve 1) was titrated with 0.34, 0.62, 0.89, and 1.09 equivalents of 4-thia-*trans*-2-octenoyl-CoA (curves 2–5, respectively). The spectral changes were complete before measurement could be made. Absorbance changes at 490 nm were best fit by a dissociation constant of 470 nM with a stoichiometry of 0.87 molecule of enoyl-CoA bound/FAD (see Materials and Methods). The inset plots difference spectra (E·L – E), where L is the thiaenoyl-CoA analogue (—) or the normal product *trans*-2-octenoyl-CoA (···), extrapolated to saturation by using a protein concentration of 16.6 μM.

hydratase effect fragmentation of the acyl chain presumably via formation of the thiohemiacetal:

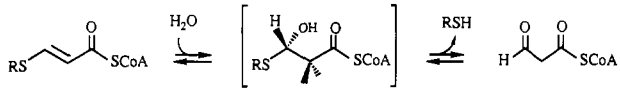


Figure 4 shows the spectral changes upon the addition of crotonase to the 4-thiaenoyl-CoA derivative at pH 7.6. The absorption band at 312 nm is replaced with one at 299 nm characteristic of the enolate of malonylsemialdehyde-CoA (see inset; Vagelos & Earl, 1959; Thorpe, 1986). This new band is pH dependent, being virtually absent at pH 3 and fully formed at pH 10 (data not shown). Quantitating malonylsemialdehyde-CoA spectrophotometrically (Figure 4) yields a stoichiometry of 1.1 of this fragment/mol of 4-thiaenoyl-CoA. The conversion of 4-thia-*trans*-2-octenoyl-CoA into malonylsemialdehyde-CoA was also shown by HPLC using a reverse-phase column and a malonylsemialdehyde-CoA standard prepared from the hydration of propionyl-CoA (Thorpe, 1986; see Materials and Methods).

Thiol release (see above) was apparent by odor and was quantitated by running the hydration reaction in the presence of DTNB (see Materials and Methods). This experiment is feasible because crotonase is only slowly inactivated by relatively high concentrations of DTNB (Waterson et al., 1972). No detectable breakdown of the enoyl-CoA occurs in the absence of enzyme as expected from the stability of this species (see Materials and Methods). However, 1.05 thiol is released/mol of 4-thia-*trans*-2-enoyl-CoA in the presence of crotonase.

To date, no spectrophotometric evidence has been obtained for the thiohemiacetal that would be the expected intermediate in the hydration of an enoyl-CoA derivative (see above). However, such thiohemiacetals would be expected to equilibrate very rapidly in dilute aqueous solution to aldehyde and thiol. Attempts to trap the thiohemiacetal in the presence of 3-OH-acyl-CoA dehydrogenase and NAD^+ were unsuccessful (data not shown). We do not know, of course, whether such species would even be substrates of this pyridine nucleotide linked dehydrogenase.

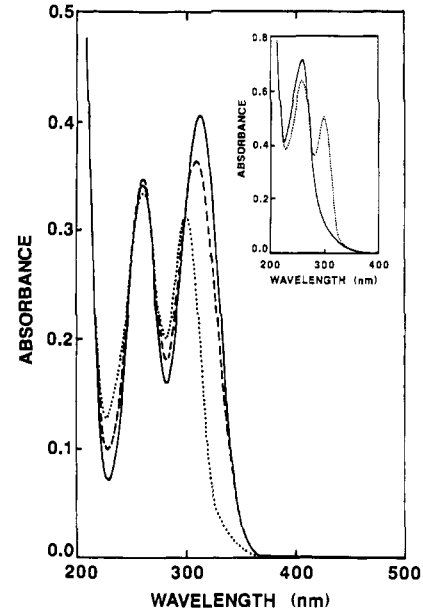


FIGURE 4: Spectral changes observed upon hydration of 4-thia-*trans*-2-octenoyl-CoA by crotonase. 4-Thia-*trans*-2-octenoyl-CoA (18 μM in 0.85 mL of 50 mM phosphate buffer, pH 7.6, at 25 °C; —) was mixed with 40 μg of crotonase, and the spectra were recorded after 1 (- - -) and 41 min (···). The inset shows the spectrum of 35.5 μM 2-propynoyl-CoA in 1.0 mL of 50 mM phosphate buffer, pH 7.6, before (—) and after (···) the addition of 20 μg of crotonase to generate malonylsemialdehyde-CoA (see text).

4-Thiaenoyl-CoA Derivatives as Substrates of Crotonase. These analogues are comparatively poor substrates of crotonase. Thus, 4-thia-*trans*-2-pentenoyl-CoA shows a V_{max} of only 0.6% of the rate shown by crotonyl-CoA (see Materials and Methods) but would still have a turnover of over 2000/min (Stern, 1961; Waterson & Hill, 1972). Under these conditions, the K_m observed for the analogue is 17 μM compared to 49 μM measured for crotonyl-CoA. Previous work has shown that crotonylpantetheine is a very poor substrate of crotonase (0.013% that of the CoA thioester; Stern & del Campillo, 1956; Stern, 1961). In view of the above, we tested (4-thia-*trans*-2-octenoyl)pantetheine and found no spectral change upon incubation of 6.4 μM thioester with 10 units of crotonase for 30 min. If this enoylpantetheine derivative is a substrate of the hydratase, it must be a very poor one.

Preparation and Properties of 4-Oxa-trans-2-octenoyl-CoA. We were unable to prepare 4-oxaoctenoyl-CoA using acyl-CoA oxidase because 4-oxaoctanoyl-CoA is a very poor substrate of this enzyme (turnover number about 0.5% of octanoyl-CoA in the standard assay; Jiang & Thorpe, 1983). However, the unsaturated analogue was obtained by incubation with medium-chain acyl-CoA dehydrogenase using ferricenium hexafluorophosphate as terminal oxidant (see Materials and Methods). 4-Oxa-*trans*-2-octenoyl-CoA was purified by HPLC, eluting after the corresponding saturated analogue as observed with the 4-thia derivatives (18.7 vs 15.8 min respectively; see Materials and Methods). The spectrum of the 4-oxaenoyl thioester is shown in Figure 5 (λ_{max} = 264 nm with an extinction coefficient of 23.7 mM^{-1} cm^{-1}). The 4-oxaenoyl chromophore would be expected to be about 50 nm blue shifted (Silverstein et al., 1981) from the 312-nm absorbance of 4-thia-*trans*-2-enoyl thioesters shown in Figure 1. Accordingly, the 4-oxaenoyl and adenine chromophores overlap, leading to the apparently single peak at 264 nm observed in Figure 5.

4-Oxa-*trans*-2-octenoyl-CoA is also a substrate of crotonase yielding malonylsemialdehyde-CoA (Figure 5) as observed with the corresponding thia analogue. In the present case

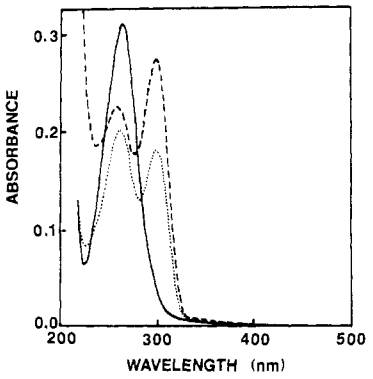


FIGURE 5: Spectrum of 4-oxa-*trans*-2-octenoyl-CoA and hydration by crotonase. 4-Oxa-*trans*-2-octenoyl-CoA (12.9 μM in 0.7 mL of 50 mM phosphate buffer, pH 7.6, 25 °C; —) was hydrated with 40 μg of crotonase for 5 min (···). The solution was then brought to pH 11 by the addition of 20 μL of KOH and the spectrum of the fully formed enolate recorded (- - -; Thorpe, 1986).

butanol is the expected other product of the nonenzymatic decomposition of the hemiacetal and was quantitated by using alcohol dehydrogenase and NAD^+ (0.88 molecule of alcohol/malonylsemialdehyde-CoA liberated; see Materials and Methods).

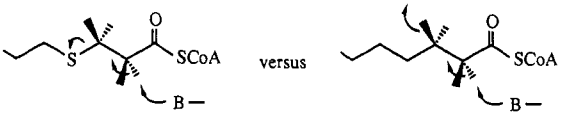
Discussion

This paper introduces 4-thia- and 4-oxaenoyl-CoA derivatives as interesting analogues in the study of β-oxidation enzymes. The chromophoric properties of the 4-thia analogues make them potentially very useful in the development of new assays of the acyl-CoA oxidases and dehydrogenases (Lehman and Thorpe, unpublished observations). Further, the large red shift (of about 25 nm) encountered when 4-thia-*trans*-2-octenoyl-CoA is bound to the medium-chain acyl-CoA dehydrogenase suggests that these enoyl-CoA derivatives might also serve as active-site probes for other β-oxidation enzymes.

The origin of this sizable spectral perturbation is not understood. It is not simply due to transfer of the chromophore from bulk solvent to a region of lower polarity because this would lead to a blue shift upon binding (Silverstein et al., 1981). The absorbance maximum of (4-thia-*trans*-2-octenoyl)pantetheine in water and acetonitrile confirms this prediction (312 vs 302 nm, respectively; Figure 1 and data not shown). Possibly, the red shift reflects a strong polarization of the thioester carbonyl, e.g., by an adjacent positive charge that could serve to stabilize the developing enolate during catalysis (Thorpe, 1987). A similar red shift of the enone chromophore of the competitive inhibitor 19-nortestosterone upon binding to Δ^5-3-ketosteroid isomerase (Wang et al., 1963; Kuliopulos et al., 1989) has been attributed to a protonation of the steroid carbonyl by tyrosine-14 in this enzyme (Kuliopulos et al., 1989).

Although the 4-thia-substituted acyl chain would be expected to be slightly longer (Weast & Astle, 1979) and more polar (Hansch & Leo, 1979; Heukeroth et al., 1988) than its hydrocarbon parent, replacement of a methylene group by a sulfur atom appears to be a reasonably conservative change in the systems studied here. Thus, 4-thiaoctanoyl-CoA is an excellent substrate of the medium-chain acyl-CoA dehydrogenase and a competent reductant of the enzyme in stopped-flow experiments (Figure 2). Thermodynamically, the thia derivative is a slightly better reductant than octanoyl-CoA (by about 35 mV, see earlier). Both normal and substituted substrates effect very similar spectral changes with reduction of the flavin chromophore and the appearance of a band at about 570 nm ascribed to a charge-transfer complex between reduced flavin as the donor and enoyl-CoA as the acceptor (Lau et al., 1988a). Similarly, *trans*-2-octenoyl-CoA and its 4-thia counterpart produce very similar perturbation of the oxidized flavin chromophore (Figure 3) with K_d values differing by about 5-fold (470 vs 90 nM; Powell et al., 1987). Finally we note that substitution of a sulfur atom at the 3-position of the thioether octyl-SCoA does not lead to a significant change in the binding affinity to the medium-chain dehydrogenase (3.6 and 4.0 μM, respectively; Lau et al., 1988b).

At the outset of these studies with 4-thiaoctanoyl-CoA, it appeared possible that α-proton abstraction would result in elimination of an alkylthiolate rather than the hydride



in analogy to the fluoride elimination observed upon incubation of butyryl-CoA dehydrogenase with 3-fluoropropionyl-CoA (Fendrich & Abeles, 1982). No such elimination was observed even with 3-(phenylthio)propionyl-CoA (see Materials and Methods), and in all cases hydride transfer appears to be the sole mechanism for the discharge of the developing enolate.

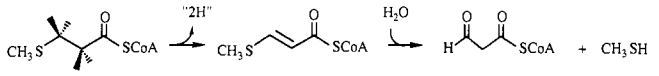
4-Thiaoctanoyl-CoA reduces the medium-chain enzyme somewhat slower than octanoyl-CoA in the reductive half-reaction (Figure 2), although both analogues reduce the flavin to similar extents (Lau et al., 1988b). Importantly, the rate constant for the rise in absorbance at 340 nm, which reflects the formation of enzyme-bound 4-thiaenoyl-CoA (see earlier), is essentially the same as that observed for the reduction of the flavin (at 446 nm) and the appearance of the long-wavelength band at 570 nm. Similarly, stopped-flow experiments by McFarland and co-workers have shown that furylacryloyl-CoA is formed at the same rate as reduction of the medium-chain enzyme by furylpropionyl-CoA (McFarland et al., 1982). Thus, in these two examples, reduction of the flavin is accompanied by the appearance of enoyl-CoA product. These observations, together with other lines of evidence (Pohl et al., 1986; Lau et al., 1988a; Schopfer et al., 1988), contradict a claim that these purple enzyme complexes represent a form of supramolecular resonance species with neither flavin nor thioester in a definable redox state (Ikeda et al., 1985). Since the data on which this suggestion was made appear to have been misinterpreted (Lau et al., 1988a), there is apparently no reliable evidence to contradict the consensus that these purple species are charge-transfer complexes between reduced flavin and enoyl-CoA product (Massey & Ghisla, 1974).

In contrast to the above, oxa substituents at either the 3- or 4-position appear to be less satisfactory analogues for acyl-CoA oxidase and dehydrogenase. This may reflect, in part, the increased polarity of the oxa substituent compared to thia or carba substituents (compare, e.g., the HPLC elution time of 4-oxaoctanoyl-CoA with that of 4-thiaoctanoyl-CoA and octanoyl-CoA of 15.8, 19.3, and 22.3 min, respectively). 3-Oxaoctanoyl-CoA binds some 25-fold more weakly than the corresponding thia derivative to the medium-chain dehydrogenase (Lau et al., 1988b). 4-Oxaoctanoyl-CoA is a very poor substrate of the oxidase and shows a rate of only about 10% of that observed with 4-thiaoctanoyl-CoA in the standard dehydrogenase assay (see above). These differences are accentuated in the reductive half-reaction of the acyl-CoA dehydrogenase. Thus, the 4-oxa analogue reduces acyl-CoA dehydrogenase in the stopped-flow apparatus at a rate of only 0.4 s^{-1} at 1 °C, more than 2 orders of magnitude slower than

for the corresponding phase when 4-thiaoctanoyl-CoA is used. In addition to polarity effects, the inductive effect of the 4-oxa moiety would be expected to render hydride transfer less favorable. However, the relative contributions of polarity, electronic, and steric effects to the drastic slowing of the reductive half-reaction with 4-oxaoctanoyl-CoA cannot be determined at present. It should be noted that previous attempts to correlate the rate of reduction of the enzyme with substituent effects for an extensive series of dihydrocinnamoyl-CoA derivatives failed decisively (Murfin, 1974). Evidently these rates were dominated by unanticipated factors unrelated to the expected electronic effects at the β-carbon (Murfin, 1974).

The work with 4-thiaalkyl-CoA derivatives provides a plausible mechanism for the release of methanethiol during methionine catabolism. In addition to the well-recognized transulfuration sequence (Cooper, 1983), a second pathway has been proposed (Benevenga, 1984; Livesey, 1984; Scislowski et al., 1987) in which L-methionine is converted to 4-(methylthio)-2-oxobutyrate by transamination (Cooper & Meister, 1976). D-Methionine may also yield this derivative upon oxidation with D amino acid oxidase (Meister & Wellner, 1963; Huxtable, 1986; Kagi et al., 1980). Oxidative decarboxylation of 4-(methylthio)-2-oxobutyrate yields 3-(methylthio)propionyl-CoA (Livesey, 1984; Scislowski et al., 1987; Benevenga & Haas, 1987), although the subsequent steps leading to the release of toxic methanethiol have not been clearly defined.

The present work shows that 3-(methylthio)propionyl-CoA (4-thiapentanoyl-CoA) is a substrate of both medium- and short-chain acyl-CoA dehydrogenase and acyl-CoA oxidase. For example, 4-thiapentanoyl-CoA and butyryl-CoA are dehydrogenated at 26% and 10%, respectively, of the rate shown by octanoyl-CoA in the standard assay of the medium-chain acyl-CoA dehydrogenase (using 30 μM substrate; Thorpe, 1981). The short-chain acyl-CoA dehydrogenase oxidizes 4-thiapentanoyl-CoA at 27% of the rate given by butyryl-CoA (using 50 μM substrates; see Materials and Methods). Hydration of the corresponding enoyl-CoA product would lead to the release of methanethiol and malonylsemialdehyde-CoA:



Whether this series of reactions actually contributes to the toxic effects of excess methionine in vivo must await further work. It will also be interesting to explore the metabolic fate and possible utility of thia fatty acids substituted at even-numbered positions to determine whether thiol release can occur in vivo.

Acknowledgments

We thank Nicole Nataro for a gift of short-chain acyl-CoA dehydrogenase and Jing Zhi Zhou for help with the stopped-flow measurements.

References

Benevenga, N. J. (1984) *Adv. Nutr. Res. 6*, 1–18.

Benevenga, N. J., & Haas, L. G. (1987) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 46*, 572.

Bernert, J. T., & Sprecher, H. (1977) *J. Biol. Chem. 252*, 6736–6744.

Cooper, A. J. L. (1983) *Annu. Rev. Biochem. 52*, 187–222.

Cooper, A. J. L., & Meister, A. (1976) *Biochemistry 11*, 661–671.

Corkey, B. E., Brandt, M., Williams, R. J., & Williamson, J. R. (1981) *Anal. Biochem. 118*, 30–41.

Degani, Y., & Patchornik, A. (1971) *J. Org. Chem. 36*, 2727–2728.

Fendrich, G., & Abeles, R. H. (1982) *Biochemistry 21*, 6685–6695.

Freund, K., Mizzer, J. P., Dick, W., & Thorpe, C. (1985) *Biochemistry 24*, 5996–6002.

Ghisla, S., Thorpe, C., & Massey, V. (1984) *Biochemistry 23*, 3154–3160.

Gorelick, R. J., Mizzer, J. P., & Thorpe, C. (1982) *Biochemistry 21*, 6936–6942.

Gorelick, R., Schopfer, L., Ballou, D. P., Massey, V., & Thorpe, C. (1985) *Biochemistry 24*, 6930–6839.

Hall, C. L., Lambeth, J. D., & Kamin, H. (1979) *J. Biol. Chem. 254*, 2023–2031.

Hansch, C., & Leo, A. (1979) in *Substituent Constants for Correlation Analysis in Chemistry and Biology*, pp 13–37, Wiley-Interscience, New York.

Heukeroth, R. O., Glaser, L., & Gordon, J. I. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 8795–8799.

Huxtable, R. (1986) in *Biochemistry of Sulfur*, Plenum Press, New York.

Ikeda, Y., Hine, D. G., Okamura-Ikeda, K., & Tamaka, K. (1985b) *J. Biol. Chem. 260*, 1326–1337.

Jiang, Z.-Y., & Thorpe, C. (1983) *Biochemistry 22*, 3752–3758.

Kaji, H., Saito, N., Murao, M., Ishimoto, M., Kondo, H., Gasa, S., & Saito, K. (1980) *J. Chromatogr. 221*, 145–148.

Kawaguchi, A., Tsubotani, S., Seyama, Y., Yamakawa, T., Osumi, T., Hashimoto, T., Kikuchi, T., Ando, M., & Okuda, S. (1980) *J. Biochem. 88*, 1481–1486.

Kuliopulos, A., Mildvan, A. S., Shortle, D., & Talalay, P. (1989) *Biochemistry 28*, 149–159.

Lau, S. M., & Thorpe, C. (1988a) *Arch. Biochem. Biophys. 262*, 293–297.

Lau, S.-M., Brantley, R., & Thorpe, C. (1988b) *Biochemistry 27*, 5089–5095.

Livesey, G. (1984) *Trends Biochem. Sci. 9*, 27–29.

Massey, V., & Ghisla, S. (1974) *Ann. N.Y. Acad. Sci. 227*, 446–465.

McFarland, J. T., Lee, M.-Y., Reinsch, J., & Raven, W. (1982) *Biochemistry 21*, 1224–1229.

McKean, M. C., Frerman, F. E., & Mielke, D. M. (1979) *J. Biol. Chem. 254*, 2730–2735.

Meister, A., & Wellner, D. (1963) *Enzymes, 2nd Ed.*, 609–648.

Murfin, W. W. (1974) Ph.D. Thesis, Washington University, St. Louis, MO.

Pohl, B., Raichle, T., & Ghisla, S. (1986) *Eur. J. Biochem. 160*, 109–115.

Powell, P. J., Lau, S.-M., Killian, D., & Thorpe, C. (1987) *Biochemistry 26*, 3704–3710.

Schopfer, L. M., Massey, V., Ghisla, S., & Thorpe, C. (1988) *Biochemistry 27*, 6599–6611.

Scislowski, P. W. D., Hokland, B. M., Davis-van Thienen, W. I. A., Bremer, J., & Davis, E. J. (1987) *Biochem. J. 247*, 35–40.

Shaw, L., & Engel, P. C. (1984) *Biochem. J. 128*, 511–520.

Silverstein, R. M., Bassler, G. C., & Morrill, T., C. (1981) in *Spectrometric Identification of Organic Compounds*, 4th ed., pp 305–331, Wiley, New York.

Stadtman, E. R. (1957) *Methods Enzymol. 3*, 931–941.

Stankovich, M. T., & Soltysik, S. (1987) *Biochemistry 26*, 2627–2632.

Stern, J. R. (1961) *Enzymes, 2nd Ed. 5*, 511–529.

Stern, J. R., & del Campillo, A. (1956) *J. Biol. Chem. 218*, 985–1002.
Steyn-Parvé, E. P., & Beinert, H. (1958) *J. Biol. Chem. 233*, 843–852.
Thorpe, C. (1981) *Methods Enzymol. 71*, 366–374.
Thorpe, C. (1986) *Anal. Biochem. 155*, 391–394.
Thorpe, C. (1987) in *Flavins and Flavoproteins* (Edmondson, D. E., & McCormick, D. B., Eds.) pp 149–157, de Gruyter, New York.
Thorpe, C., Matthews, R. G., & Williams, C. H. (1979) *Biochemistry 18*, 331–337.
Vagelos, P. R., & Earl, J. M. (1959) *J. Biol. Chem. 234*, 2272–2279.
Wang, S. F., Kawahara, F. S., & Talalay, P. (1963) *J. Biol. Chem. 238*, 576–585.
Waterson, R. M., & Hill, R. L. (1972) *J. Biol. Chem. 247*, 5258–5265.
Waterson, R. M., Hass, G. M., & Hill, R. L. (1972) *J. Biol. Chem. 247*, 5252–5257.
Weast, R. C., & Astle, M. J. (1979) *CRC Handbook of Chemistry and Physics*, 60th ed., pp F216–F219, CRC, Boca Raton, FL.

CORRECTION

Amino Acid Sequence of Bovine Angiogenin, by Michael D. Bond and Daniel J. Strydom*, Volume 28, Number 14, July 11, 1989, pages 6110–6113.

Page 6112. Figure 2 should appear as follows.

```
                1                   10                  20                  30
RNase A           k E t a A a K F e R Q H m D s s t s a a s s s n Y C N q M M k
Bov. Ang.   A Q D D Y R Y I H F L T Q H Y D A K P K G R N D E - Y C F N M M K
Hum. Ang.     Q D N S R Y T H F L T Q H Y D A K P Q G R D D R - Y C E S I M R

                        40                  50                  60
RNase A     s R n l T k d r C K P V N T F v H E s l a d V q A v C s Q k n V a
Bov. Ang.   N R R L T R P - C K D R N T F I H G N K N D I K A I C E D R N G Q
Hum. Ang.   R R G L T S P - C K D I N T F I H G N K R S I K A I C E N K N G N

                    70                  80                  90
RNase A     C K N g q t N C y q S y s t m s I T d C R e t g s S k y P n C a Y
Bov. Ang.   P Y R G - - D L R I S K S E F Q I T I C K H K G G S S R P P C R Y
Hum. Ang.   P H R E - - N L R I S K S S F Q V T T C K L H G G S P W P P C Q Y

                100                 110                 120
RNase A     k t t q a n k h I i v A C e G n P y V P V H f D a s V
Bov. Ang.   G A T E D S R V I V V G C E N G - - L P V H F D E S F I T P R H
Hum. Ang.   R A T A G F R N V V V A C E N G - - L P V H L D Q S I F R R P
```