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Spatial Arrangement of Coenzyme and Substrates Bound to L-3-Hydroxyacyl-CoA Dehydrogenase As Studied by Spin-Labeled Analogues of NAD⁺ and CoA^{†,‡}

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ABSTRACT: The synthesis of nitroxide spin-labeled derivatives of *S*-acetoacetyl-CoA, *S*-acetoacetylpantetheine, and *S*-acetoacetylcysteamine is described. These compounds are active substrates of L-3-hydroxyacyl-CoA dehydrogenase [*(S)*-3-hydroxyacyl-CoA:NAD⁺ oxidoreductase, EC 1.1.1.35] exhibiting v_{\max} values from 20% to 70% of *S*-acetoacetyl-CoA itself. *S*-Acetoacetylpantetheine and *S*-acetoacetylcysteamine form binary complexes with the enzyme and exhibit ESR spectra typical for immobilized nitroxides. In the case of spin-labeled pantetheine, the radical is more mobile. When spin-labeled substrates are bound simultaneously to each active site of this dimeric enzyme, spin-spin interactions differentiate between two alternate orientations of the substrate [Birktoft, J. J., Holden, H. M., Hamlin, R., Xuong, N. H., & Banaszak, L. J. (1987) *Proc. Natl. Acad. Sci. U.S.A.* 84, 8262-8266]. The fatty acid moiety is thought to be located in a cleft between two domains whereas a large part of the CoA moiety probably extends into the solution. NAD⁺, spin-labeled at N⁶ of the adenine ring, is an active coenzyme of L-3-hydroxyacyl-CoA dehydrogenase (60% v_{\max}). Complexes with the enzyme exhibit ESR spectra typical of highly immobilized nitroxides. Binding of coenzyme NAD⁺ causes conformational changes of the binary enzyme/substrate complex as revealed by changes in the ESR spectrum of spin-labeled *S*-acetoacetylpantetheine.

L-3-Hydroxyacyl-CoA dehydrogenase [*(S)*-3-hydroxyacyl-CoA:NAD⁺ oxidoreductase, EC 1.1.1.35] catalyzes the NAD-dependent interconversion between L-3-hydroxy and 3-oxo fatty acyl CoA thioesters. The fatty acid chain length

can vary from 4 to 20 (Wakil et al., 1954). The enzyme has been isolated from various sources (Lynen et al., 1952; Wakil et al., 1954; Stern, 1957; Grassl, 1957), and more recently, the crystal structure of the dehydrogenase from pig heart mitochondria has been determined by X-ray crystallography (Birktoft et al., 1987). 3-Hydroxyacyl-CoA dehydrogenase from this source is a dimer with a molecular weight of $M_r = 67\,000$ (Bitar et al., 1980; Birktoft et al., 1987). Some heterogeneity at the amino termini of the chains may be the result of proteolytic processing. Each dimer is composed of two domains with the amino-terminal end of each domain comprising a typical Rossmann fold (Rossmann et al., 1975) for binding of the adenine moiety of NAD. Much less is

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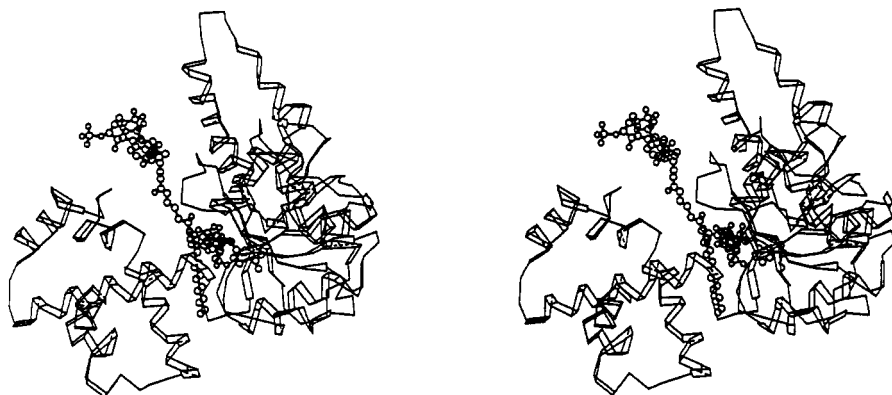


FIGURE 1: Hypothetical ternary complex of L-3-hydroxyacyl-CoA dehydrogenase. The stereo diagram shows a schematic drawing of the molecular model of the A subunit of the enzyme together with a bound NAD. The hypothetical binding mode for the fatty acyl-CoA substrate is also shown with the fatty acid moiety located in the cleft between the domains of the subunit. NAD and fatty acyl-CoA are shown as ball-and-stick models [with kind permission from Birktoft et al. (1987)].

known about the catalytic site and binding of the CoA esters. Two general arrangements for the L-3-hydroxy fatty acyl CoA molecule seem possible when it is positioned adjacent to the nicotinamide ring of the NAD such that the hydrogen on the 3-carbon of the fatty acid is near the 4-carbon of the nicotinamide ring. Assuming a fixed conformation for the substrate, this is equivalent to rotating it 180°, maintaining the position of the 3-carbon. The more favorable orientation places the fatty acid moiety in the cleft between the two domains, allowing for close contacts with the enzyme and the CoA moiety toward the more open end of the cleft as shown in Figure 1 (Birktoft et al., 1987). In the alternative orientation, the two ends of the substrate are interchanged.

Spin-labeled NAD derivatives have successfully been employed in studies of structure-function relationships of various dehydrogenases (Fritzsch et al., 1984; Glöggler et al., 1982a; Trommer & Glöggler, 1979; Trommer, 1987). In particular, valuable information about the solution structure of glyceraldehyde-3-phosphate dehydrogenase, e.g., intramolecular distances, has been obtained from dipolar spin-spin interaction observed between spin-labeled NAD molecules bound to adjacent subunits of the tetrameric enzyme (Beth et al., 1984; Glöggler et al., 1982a; Wilder et al., 1989). Enhanced sensitivity and greatly facilitated interpretation and simulation of the ESR spectra in these studies were achieved by isotopic substitution of the spin-label (Glöggler et al., 1982b; Philipp et al., 1984).

To differentiate between the two possible orientations for the substrate, spin-labeled NAD⁺ (Figure 2) and newly synthesized spin-labeled CoA esters have been used (Figure 3).

EXPERIMENTAL PROCEDURES

Materials

Pig heart L-3-hydroxyacyl-CoA dehydrogenase as an ammonium sulfate precipitate, NAD⁺, and NADH were obtained from Boehringer Mannheim (Mannheim, FRG). N⁶-SL-

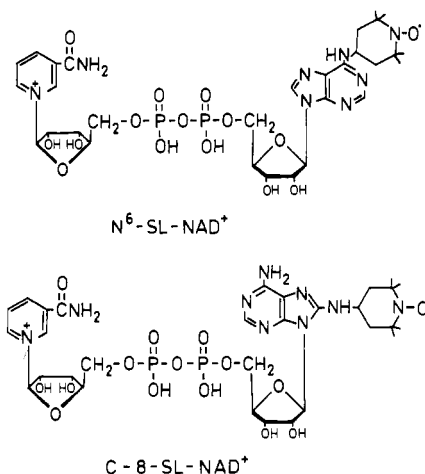


FIGURE 2: Structural formulas of the coenzyme analogues N⁶-SL-NAD⁺ and C8-SL-NAD⁺.

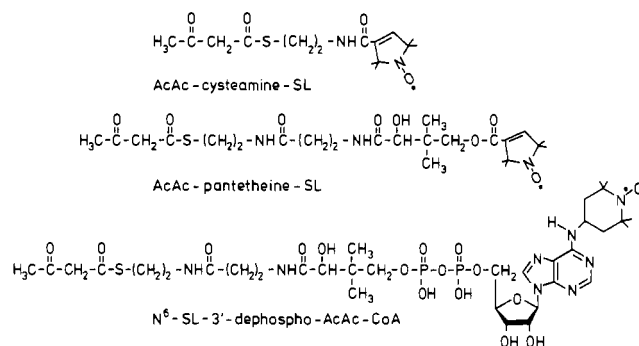


FIGURE 3: Structural formulas of the spin-labeled substrates N⁶-SL-AcAc-CoA, AcAc-pantetheine-SL, and AcAc-cysteamine-SL.

NAD⁺,¹ N⁶[¹⁵N,²H₁₇]SL-NAD⁺, and C8-SL-NAD⁺ were prepared as previously described (Trommer et al., 1974; Philipp et al., 1984; Wenzel & Trommer, 1977). 4-Amino-2,2,6,6-tetramethylpiperidine-1-oxyl was obtained from Aldrich-Chemie (Steinheim, FRG) and 2,2,5,5-tetramethyl-1-oxy-3-pyrroline-3-carboxylic acid from Eastman Kodak (Rochester, NY).

ESR spectra were recorded with a Bruker ESP 300 spectrometer operating in the X-band mode at 100-kHz modulation with a microwave power of 6.3 mW and a peak-to-peak modulation amplitude of 1 G. Measurements were carried out at 24 °C in micro flat cells (Wenzel et al., 1976) in a total volume of 50 μL. L-3-Hydroxyacyl-CoA dehydrogenase was centrifuged at 34000g prior to the ESR experiments, and the

¹ Abbreviations: N⁶-SL-NAD⁺, nicotinamide N⁶-(2,2,6,6-tetramethyl-1-oxypiperidin-4-yl)adenine dinucleotide; N⁶-[¹⁵N,²H₁₇]SL-NAD⁺, nicotinamide N⁶-(¹⁵N,²H₁₇)-2,2,6,6-tetramethyl-1-oxypiperidin-4-yl)adenine dinucleotide; C8-SL-NAD⁺, nicotinamide 8-[(2,2,6,6-tetramethyl-1-oxypiperidin-4-yl)amino]adenine dinucleotide; CoA, coenzyme A; N⁶-SL-3'-dephospho-CoA, N⁶-[(2,2,6,6-tetramethyl-1-oxypiperidin-4-yl)-3'-dephospho]coenzyme A; AcAc preceding CoA, cysteamine, or pantetheine refers to their S-acetoacetyl derivatives; SL as suffix to cysteamine refers to its 2,2,5,5-tetramethyl-1-oxy-3-pyrroline-3-carboxylic acid amide; SL as suffix to pantetheine refers to its 4'-(2,2,5,5-tetramethyl-1-oxy-3-pyrroline-3-carboxylic acid ester); EDTA, ethylenediaminetetraacetic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

pellet was redissolved in 250 mM Tris-HCl buffer, pH 7.2, or 100 mM sodium pyrophosphate buffer, pH 6.8, both containing 0.2% EDTA. Further conditions are given in the legends to the figures. For the determination of binding constants by ESR titrations the spin-labeled substrates were varied in the following concentration ranges: SL-AcAc-cysteamine from 50 μ M to 1 mM at 220 to 370 μ M enzyme; SL-AcAc-pantetheine from 150 μ M to 1 mM at 250 to 370 μ M enzyme; N^6 -SL-AcAc-CoA from 15 to 150 μ M at 80 μ M enzyme. Binding of N^6 -SL-NAD⁺ was determined between 89 to 400 μ M of the coenzyme analogue and 110 to 150 μ M enzyme.

Enzyme assays were carried out according to Decker (1955) at 25 °C in 100 mM phosphate buffer by following the decrease of the NADH (250 μ M) absorption at 365 nm in the presence of 100 μ M AcAc-CoA. In the case of SL-AcAc-pantetheine and SL-AcAc-cysteamine, the NADH concentration was reduced to 50 μ M and the concentration of the substrates to 50 μ M. Relative activities of the substrate analogues as given under Results are, however, based on data obtained with the natural substrates at identical concentrations.

Protein concentrations were determined by the method of Lowry et al. (1951) and are based on $M_r = 35\,000$ (Bitar et al., 1980). Specific activities ranged from 90 to 120 units/mg.

Molecular Modeling

L-3-Hydroxyacyl-CoA dehydrogenase crystal coordinates were taken from Birktoft et al. (1987). α atoms were displayed on an IRIS 4D work station (Silicon Graphics, Mountain View, CA) employing the program QUANTA (version 2.1A, Polygen, Waltham, MA). The conformations of SL-AcAc-pantetheine and SL-AcAc-cysteamine in vacuo were determined by force-field calculations employing the program CHARMM 20.3.2 (Polygen, Waltham, MA). A carbonyl group was substituted for the nitroxide in these calculations. The molecules were then fitted with the fatty acid moiety into the cleft between the two domains of the L-3-hydroxyacyl-CoA dehydrogenase monomers in such a way as to allow for hydride transfer from the nicotinamide ring as postulated by Birktoft et al. (1987).

Synthetic Procedures

Thin-layer chromatography was carried out on precoated silica plates, 60F-254 (E. Merck, Darmstadt, FRG), with the following systems as eluents: system I, butan-1-ol/acetic acid/water (5:2:3 by volume) and system II, propan-2-ol/ammonia/water (7:1:2 by volume).

AcAc-Cysteamine-SL. Cysteamine was thioacylated with diketene according to a general procedure by Noyes and Bradshaw (1973). Freshly distilled diketene (150 μ L) was dissolved in 2 mL of 100 mM aqueous potassium bicarbonate (pH 8.5) and was then added dropwise under stirring to an ice-cold solution of 56 mg (0.5 mmol) of cysteamine hydrochloride in 2 mL of water. After completion of the reaction (about 10 min) as monitored in aliquots by determination of remaining free SH groups according to Ellman (1959), the pH of the reaction mixture was taken to 5.0 with 2 N HCl. Unreacted diketene was removed by extraction with dry ethyl ether (three times with 5 mL), and the aqueous phase was concentrated under reduced pressure to about 1 mL. This solution was used for the subsequent esterification without further purification.

Ninety-two milligrams (0.5 mmol) of 2,2,5,5-tetramethyl-1-oxy-3-pyrroline-3-carboxylic acid and 50 mg (0.5 mmol) of triethylamine in 2 mL of ethyl ether were converted to a mixed anhydride according to Griffith et al. (1967) by

addition of a solution of 27 mg (0.5 mmol) of ethyl chloroformate in 2 mL of ethyl ether. The ether was removed under reduced pressure and the residue recrystallized from hexane, yielding the mixed anhydride in 86% yield (110 mg).

The crude AcAc-cysteamine in 1 mL of water was added under stirring to a solution of 128 mg (0.5 mmol) (determined by UV at 300 nm, $\epsilon = 18\,800$, pH 8.0) of the mixed anhydride in freshly distilled dry dimethylformamide according to the procedure of Lynen et al. (1952). Stirring was continued for 2 h, and the reaction mixture was then concentrated to about 1 mL under reduced pressure. This crude product was purified by gel chromatography on a Sephadex LH-20 column (1 \times 100 cm) with water as eluent. AcAc-cysteamine-SL was eluted after 92 mL as the second of four fractions (25 mg, 14%). UV: λ_{\max} at pH 7.0, 280 and 240 nm; at pH 9.0, 279 nm typical for the acetothiolacetate moiety (Decker, 1955). Both spectra show a shoulder at 300 nm resulting from the enol form. IR: amide band at 1620 cm^{-1} . TLC: $R_f = 0.63$ in solvent system I.

AcAc-Pantetheine-SL. AcAc-pantetheine was obtained by the method of Noyes and Bradshaw (1973). Pantethine (554 mg, 1 mmol) was dissolved in 2 mL of water, the pH was adjusted to 9.0 with KOH, and the disulfide bond was reduced by addition of an ice-cold solution of 5 mL of 1.5 M sodium borohydride in water. The mixture was kept at 37 °C for 15 min, cooled rapidly, and brought to pH 7.2 with 2 N HCl. A solution of 300 μ L (3.6 mmol) of freshly distilled diketene in 8 mL of aqueous 100 mM KHCO_3 , pH 8.5, was added under vigorous stirring. After completion of the reaction as monitored by with Ellman's reagent (Ellman, 1959), the pH of the solution was brought to 5.0 with HCl. Unreacted diketene was removed by repeated extraction with ethyl ether. The crude product was purified by gel chromatography on a Sephadex LH-20 column (1 \times 100 cm) with water as eluent. AcAc-pantetheine eluted as the second of two fractions after 75 mL of the eluent yielding 300 mg (54%) following lyophilization as identified by ^1H NMR (Borden, 1975). The spectrum corresponds to that of pantetheine with additional signals at 2.1 (singlet) and 3.4 ppm (multiplet arising from the keto form). This material was used without further purification for the subsequent spin-labeling. Concentrations were determined spectrophotometrically (Decker, 1955) at 237 nm, $\epsilon = 4500$ (pH 7.0), and at 300 nm, $\epsilon = 18\,800$ (pH 8.0).

To 92 mg (0.5 mmol) of 2,2,5,5-tetramethyl-1-oxy-3-pyrroline-3-carboxylic acid in 2 mL of dry dimethylformamide was added 81 mg (0.5 mmol) of carbonyldiimidazole at room temperature. After 30 min, a solution of 181 mg (0.5 mmol) of AcAc-pantetheine in 1 mL of water was added, and the mixture was stirred for 6 h. The solvent was removed under vacuum, and the oily residue was triturated three times with 5 mL of dry ethyl ether and was then dissolved in 3 mL of propan-2-ol. AcAc-pantetheine-SL precipitated upon addition of 50 mL of dry ethyl ether as a yellow powder. Further washings with ethyl ether yielded 36 mg (12%) that was kept under nitrogen. UV: λ_{\max} at pH 7.0, shoulder at 240 nm; at pH 8.0, 300 nm; at pH 9.0, 304 nm. IR: amide band at 1650 cm^{-1} . TLC: $R_f = 0.78$ in solvent system I.

N^6 -SL-3'-dephospho-AcAc-CoA. D-Pantetheine 4'-phosphate was obtained from pantethine by the method of Moffatt and Khorana (1961a). It was first converted to the free acid by passing through an Amberlite IR 120 column (H^+ form, 1 \times 4 cm) with water as eluent and subsequently to the pyridinium salt by dissolving in an excess of pyridine and lyophilization. For ^1H NMR data, see Ciardelli et al. (1981). N^6 -SL-AMP (50 mg, 70 μ mol) (Glöggler et al., 1981) was

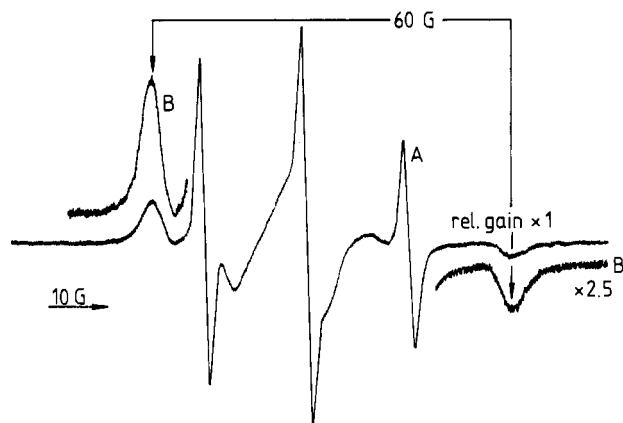


FIGURE 4: Trace A: ESR spectra of 165 μM N^6 -SL- NAD^+ in complex with 330 μM L-3-hydroxyacyl-CoA dehydrogenase at pH 7.3 in Tris-HCl buffer. Trace B: The immobilized component from enzyme-bound N^6 -SL- NAD^+ accounting for about 90% of the total spin-labeled coenzyme.

converted to its morpholidate with 65 mg (30 μmol) of dicyclohexylcarbodiimide and 25 μL (30 μmol) of morpholine according to the general procedure of Moffatt and Khorana (1961b).

Fifty-one milligrams (90 μmol) of pantetheine 4'-phosphate (pyridinium salt) and 26 mg (30 μmol) of N^6 -SL-AMP-morpholidate were dissolved in 4 mL of anhydrous pyridine and stirred for 4 days at room temperature. The solvent was removed under vacuum and the product purified by anion-exchange chromatography on a DEAE-cellulose column (Whatman No. 52, chloride form, 2×15 cm) with a linear salt gradient. The mixing vessel contained 1000 mL of 3 mM HCl and the reservoir 40 mM LiCl in 3 mM HCl (1000 mL). N^6 -SL-3'-dephospho-CoA eluted as the main peak at 30 mM LiCl. Lyophilization and repeated precipitation from propan-2-ol with ethyl ether and washings with acetone (3×3 mL) and finally ethyl ether yielded 5 mg (20%). Gel filtration over Sephadex G-10 (2×90 cm, water as eluent) can be substituted for repeated precipitation. Conversion to the acetothiolacetate (N^6 -SL-AcAc-CoA) was carried out according to a method by Noyes and Bradshaw (1973) in 0.25 M Tris-HCl buffer (7.2) with 3 μL (6 μmol) of diketene in 0.2 mL of 0.1 M aqueous KHCO_3 . Excess diketene was removed by repeated extraction with ethyl ether, and the solution was used without further purification in the kinetic and ESR experiments. UV: λ_{max} at pH 7.2, 274 nm. TLC: $R_f = 0.18$ in solvent system I; $R_f = 0.28$ in system II.

RESULTS

Binding of Spin-Labeled Coenzymes to L-3-Hydroxyacyl-CoA Dehydrogenase. N^6 -SL- NAD^+ (Figure 2) is active as a coenzyme with L-3-hydroxyacyl-CoA dehydrogenase reaching 60% v_{max} of NAD^+ itself. Hence, the spin-label does not perturb the native structure of the enzyme/coenzyme complex to any significant extent. When the spin-label is attached to C8 of the adenine ring (C8-SL- NAD^+ , Figure 2), the maximum rate was found to be 40% as compared to that of NAD^+ , but the binding constant is considerably reduced (see below).

N^6 -SL- NAD^+ exhibits highly immobilized ESR spectra in complexes with L-3-hydroxyacyl-CoA dehydrogenase ($A_{zz} = 60$ G; Figure 4). The line shape of this immobilized component was independent of the excess of N^6 -SL- NAD^+ employed. Binding of N^6 -SL- NAD^+ was quantitated by ESR spectroscopy from the decrease in the high-field signal amplitude of the unbound, freely tumbling species. The con-

Table I: Comparison of Kinetic and Thermodynamic Parameters of Spin-Labeled Ligands of L-3-Hydroxyacyl-CoA Dehydrogenase with Literature Values of Natural Ligands and Other Analogues^a

compound	K_d (mM)	K_m (mM)	% v_{max} ^b
NAD^+		0.016	100
N^6 -SL- NAD^+	0.025		60
C8-SL- NAD^+			40
AcAc-CoA		0.2 nM ^c	100
N^6 -SL-3'-dephospho-AcAc-CoA			70
AcAc-pantetheine		0.08	
AcAc-pantetheine-SL	0.1		33
AcAc-N-acetylcysteamine		10	
AcAc-N- β -alanylcysteamine		1.4	
AcAc-cysteamine-SL	1.0		20

^a K_m values were taken from literature data as shown in the text.

^b Relative v_{max} values were determined under standard assay conditions as described under Experimental Procedures. ^c Decker, 1974.

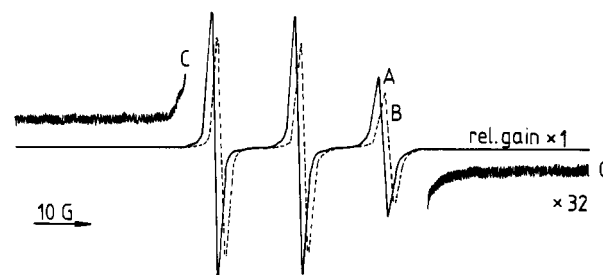


FIGURE 5: ESR spectra of 15 μM N^6 -SL-3'-dephospho-AcAc-CoA at pH 6.8 in pyrophosphate buffer in the absence (trace A) and SL compound in the presence (traces B and C) of 80 μM L-3-hydroxyacyl-CoA dehydrogenase. The center-field position in traces B and C is shifted by 1.8 G for better comparison of the spectra.

tribution from enzyme-bound N^6 -SL- NAD^+ to this signal is negligible (Griffith & Waggoner, 1969). Data were obtained at 10 different stoichiometries with ratios of N^6 -SL- NAD^+ over enzyme varied from 0.5 to 2.5 yielding $K_d = 25.5$ μM . This value correlates well with K_m of NAD^+ (15.6 μM) and with S-3-hydroxybutyryl-CoA as substrate (He et al., 1989). Binding of C8-SL- NAD^+ was found to be about 4-fold weaker (Table I).

Binding of Spin-Labeled Substrates to L-3-Hydroxyacyl-CoA Dehydrogenase. N^6 -SL-3'-dephospho-AcAc-CoA (Figure 3) closely resembles the natural substrates of L-3-hydroxyacyl-CoA dehydrogenase. It lacks the 3'-phosphate group on the adenosine moiety, which itself is not required for enzymatic activity. N^6 -SL-3'-dephospho-AcAc-CoA is reduced by NADH in the presence of the enzyme at a rate comparable to that of natural substrates (70% v_{max} , Table I). However, we have not prepared enough of the spin-label to determine all of the kinetic parameters.

ESR spectra of N^6 -SL-3'-dephospho-AcAc-CoA exhibit the typical three-line spectrum of nitroxides (Figure 5). In the presence of the enzyme (up to a 5-fold excess), the signal amplitude was reduced as compared to the spectrum of the analogue at the same concentration in buffer alone. However, the broad lines characteristic of an immobilized species could not be observed, even at very high gain. On the basis of the decrease of the signal of freely tumbling N^6 -SL-AcAc-CoA of 20%–25% at a 5-fold excess of the enzyme (36 μM), a motionally restricted component should have been detectable. However, the decrease of the signal amplitude could be reversed by the addition of a 20-fold molar excess of AcAc-CoA, indicating binding of the spin-labeled analogue at the active site. The line shape of the ESR spectra was essentially independent of pH, i.e., at 6.8 (100 mM pyrophosphate), 7.2 (250 mM Tris-HCl), or 8.5 (100 mM pyrophosphate). Tris

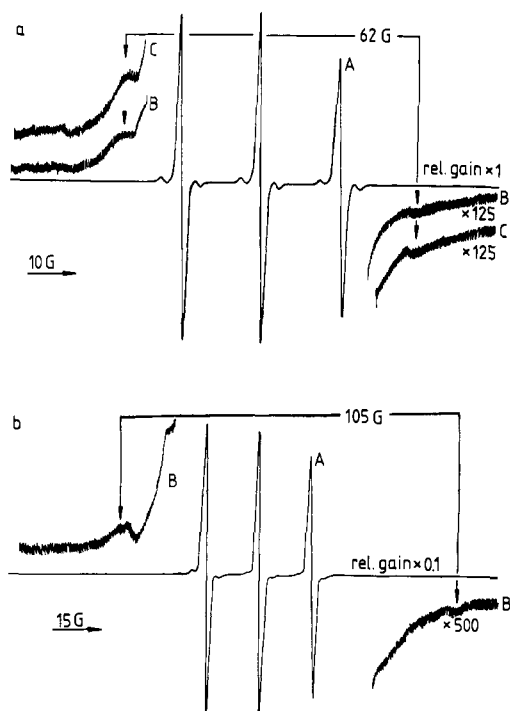


FIGURE 6: (Part a) ESR spectra (100-G scans) of AcAc-pantetheine-SL at pH 6.8 in pyrophosphate buffer. Trace A: 290 μ M AcAc-pantetheine-SL in the absence of L-3-hydroxyacyl-CoA dehydrogenase. Trace B: 290 μ M AcAc-pantetheine-SL in the presence of 370 μ M enzyme (0.8:1). Trace C: 600 μ M AcAc-pantetheine-SL in the presence of 360 μ M enzyme (1.7:1). The modulation amplitude in spectra B and C was increased to 1.2 G. (Part b) Trace A: ESR spectra (150-G scans) of 1 mM AcAc-pantetheine-SL as in part a. Trace B: 1 mM AcAc-pantetheine-SL in the presence of 250 μ M L-3-hydroxyacyl-CoA dehydrogenase (4:1). The modulation amplitude in spectrum B was increased to 1.2 G.

was reported to effect the activity of L-3-hydroxyacyl-CoA dehydrogenase (Grassl, 1957). Likewise, the addition of NAD^+ or NADH at ratios varying from 0.5 to 10 had no effect on line shape and/or binding of N^6 -SL-AcAc-CoA (23%–25% bound).

Fatty acid thioesters of CoA missing the adenine ring or even the ADP moiety of CoA were previously shown to be substrates for L-3-hydroxyacyl-CoA dehydrogenase (Grassl, 1957; Lynen & Wieland, 1955; Noyes & Bradshaw, 1973). We therefore prepared spin-labeled derivatives of AcAc-pantetheine and AcAc-cysteamine (Figure 3). These analogues could be reduced by NADH in the presence of the enzyme to the corresponding alcohols. The maximum velocities as compared to AcAc-CoA reached about 33% for AcAc-pantetheine-SL and 20% for AcAc-cysteamine-SL (Table I).

In the presence of the enzyme, both analogues exhibit ESR spectra composed of two components, arising from the freely tumbling and strongly immobilized species (Figures 6a and 7a). Employing the decrease of the signal from free spin-labeled substrates as a measure for binding as described above for N^6 -SL- NAD^+ yielded dissociation constants of about 100 μ M for AcAc-pantetheine-SL and 1 mM for AcAc-cysteamine-SL. The former correlates well with the published K_m value of 80 μ M for AcAc-pantetheine (Grassl, 1957). In the case of AcAc-cysteamine, the same author reported a K_m value of 10 mM for the *N*-acetyl derivative. However, in the corresponding amide formed by β -alanine with the amino function of AcAc-cysteamine, K_m decreased to 1.7 mM (Grassl, 1957). In AcAc-cysteamine-SL ($K_d = 1$ mM), the spin-label can be considered as being substituted for β -alanine, resulting in a

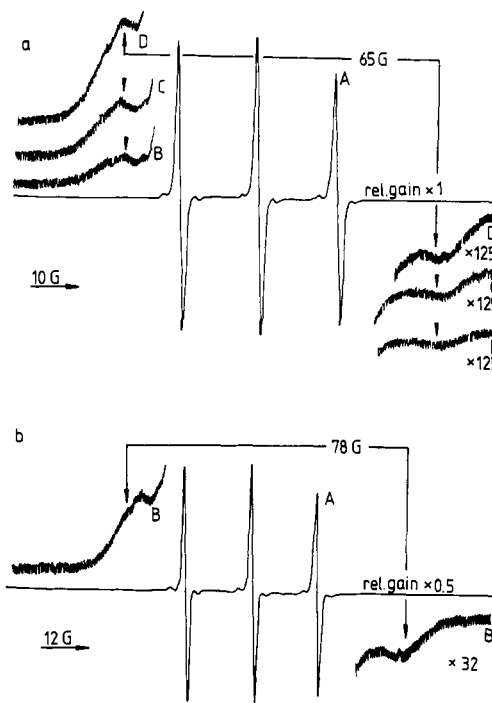


FIGURE 7: (Part a) ESR spectra (100-G scans) of AcAc-cysteamine-SL at pH 6.8 in pyrophosphate buffer. Trace A: 70 μ M AcAc-cysteamine-SL in the absence of L-3-hydroxyacyl-CoA dehydrogenase. Trace B: 70 μ M AcAc-cysteamine-SL in the presence of 370 μ M enzyme (0.2:1). Trace C: 280 μ M AcAc-cysteamine-SL and 360 μ M enzyme (0.7:1). Trace D: 530 μ M AcAc-cysteamine-SL with 350 μ M enzyme (1.5:1). In spectra B–D the modulation amplitude was increased to 1.2 G. (Part b) Trace A: ESR spectra (150-G scans) of 920 μ M SL-AcAc-cysteamine as in part a. Trace B: 920 μ M AcAc-cysteamine-SL in the presence of 370 μ M L-3-hydroxyacyl-CoA dehydrogenase (2.5:1). The modulation amplitude in spectrum B was increased to 1.2 G.

molecule having comparable length but being somewhat more bulky.

The broad ESR lines (Figures 6a and 7a), resulting from the enzyme-bound substrates, exhibit A_{zz} values of 62–63 G in the case of AcAc-pantetheine-SL and of 65 G for AcAc-cysteamine-SL. These spectra were recorded at molar ratios of substrate to enzyme of about 1. When this ratio was increased (up to 5.5), additional bands, separated by about 105 G, appeared in the low-field and high-field regions of the AcAc-pantetheine-SL spectra (Figure 6b). Such a large separation cannot be due to hyperfine interaction even when the spin-label was in an extremely polar environment or when hydrogen bonded (Damerau & Fittkau, 1983; Johnson, 1981; Humphries & McConnell, 1976). Hence, as has been observed previously (Karim et al., 1989; Wilder et al., 1989; Beth et al., 1984), the large splitting must arise from spin-spin interaction between spin-labeled substrates bound to the enzyme in close proximity. Line-shape simulations employing the program MENO by Eaton et al. (1983) for pure dipolar interaction in the rigid powder limit yielded a distance between the nitroxide groups of about 8.5–12 Å, depending on the relative geometry of the radical centers. The calculation included even extreme angles that are unlikely on the basis of molecular modeling (see Discussion) and did not account for motional narrowing as is the case in the experimental spectra. Hence, the actual distance is likely to be close to the lower limit.

In contrast to AcAc-pantetheine-SL, AcAc-cysteamine-SL, when added at molar excesses up to 5.5-fold, did not show additional bands. However, both the low-field and high-field signals became increasingly asymmetric (Figure 7b). The

maximum splitting between these shoulders reaches only 78 G and, hence, could arise from a spin-label in a highly polar environment. Alternatively, as outlined above for AcAc-pantetheine-SL, these bands could be due to spin-spin interaction between two enzyme-bound nitroxides being further apart by about 5 Å. Corresponding calculations yielded a range from 13 to 21 Å, again with the actual value being more likely in the lower range. The relative motion of the spin-label is more restricted in this case as indicated by the larger A_{zz} value in the absence of spin-spin interaction.

Ternary Complexes of Spin-Labeled Coenzymes and Spin-Labeled Substrates with L-3-Hydroxyacyl-CoA Dehydrogenase. Isotopic substitution of the spin-label has greatly enhanced the potential of the spin-labeling technique (Park & Trommer, 1989). Perdeuterated spin-labels afford higher sensitivity and better resolution due to a reduced line width. A further improvement is brought about by substituting ^{15}N for ^{14}N in the label because the nuclear manifolds are reduced from 3 to 2, resulting in different field positions of the resonance lines and an additional gain in sensitivity. It is therefore possible to differentiate between signals from ^{14}N - and ^{15}N -labeled spin probes within the same sample. Hence, when an equimolar amount of N^6 -SL- NAD^+ was added to a 1:1 mixture (based on potential binding sites) of L-3-hydroxyacyl-CoA dehydrogenase with AcAc-pantetheine-SL, a large increase in the signal amplitude of immobilized, enzyme-bound AcAc-pantetheine-SL was observed, arising from bound N^6 -SL- NAD^+ , which exhibits considerably tighter binding than the substrate (Figure 8a). Concomitantly, the A_{zz} value was reduced to 60 G as exhibited by N^6 -SL- NAD^+ alone in complex with the enzyme. However, addition of N^6 - ^{15}N , $^2\text{H}_{17}$]SL- NAD^+ caused splitting of the low-field and high-field signals of bound AcAc-pantetheine-SL (Figure 8c). The corresponding low-field signal of bound N^6 - ^{15}N , $^2\text{H}_{17}$]SL- NAD^+ overlaps partially with the low-field signal of freely tumbling ^{14}N -spin-label arising both from AcAc-pantetheine-SL and from a ^{14}N impurity (about 1%) in ^{15}N spin-labels (see arrows in Figure 8b, trace A; Karim et al., 1989). The latter impurity stems from a $^{14}\text{ND}_3$ contamination in the commercially available $^{15}\text{ND}_3$ employed in the synthesis of these labels. The high-field signal of bound N^6 - ^{15}N , $^2\text{H}_{17}$]SL- NAD^+ is well separated from other components as can be seen in trace B of Figure 8b. However, at the high-gain setting necessary to display the high-field signals from bound AcAc-pantetheine-SL, the corresponding signal from N^6 - ^{15}N , $^2\text{H}_{17}$]SL- NAD^+ is off-scale (Figure 8c, traces B and C).

The maximum separation between the outermost bands of AcAc-pantetheine-SL is about 68 G. Hence, the splitting may well be due to two distinct environments of the substrate induced by the presence of the coenzyme. This assumption was verified by the addition of unlabeled NAD^+ instead of N^6 - ^{15}N , $^2\text{H}_{17}$]SL- NAD^+ to the binary AcAc-pantetheine-SL/enzyme complex that led to the same splitting of the low-field and high-field signals of bound AcAc-pantetheine-SL.

DISCUSSION

The main question raised in this paper concerned the arrangement of the CoA ester substrates within the active center of L-3-hydroxyacyl-CoA dehydrogenase. To this end we have employed spin-labeled NAD^+ as well as various newly synthesized spin-labeled analogues of the substrate. A fundamental prerequisite for the use of spin-labeled analogues of natural ligands is that the presence of the rather bulky spin-label does not significantly alter their binding and/or catalytic constants. This condition has been adequately met in the present investigation as both the spin-labeled coenzyme

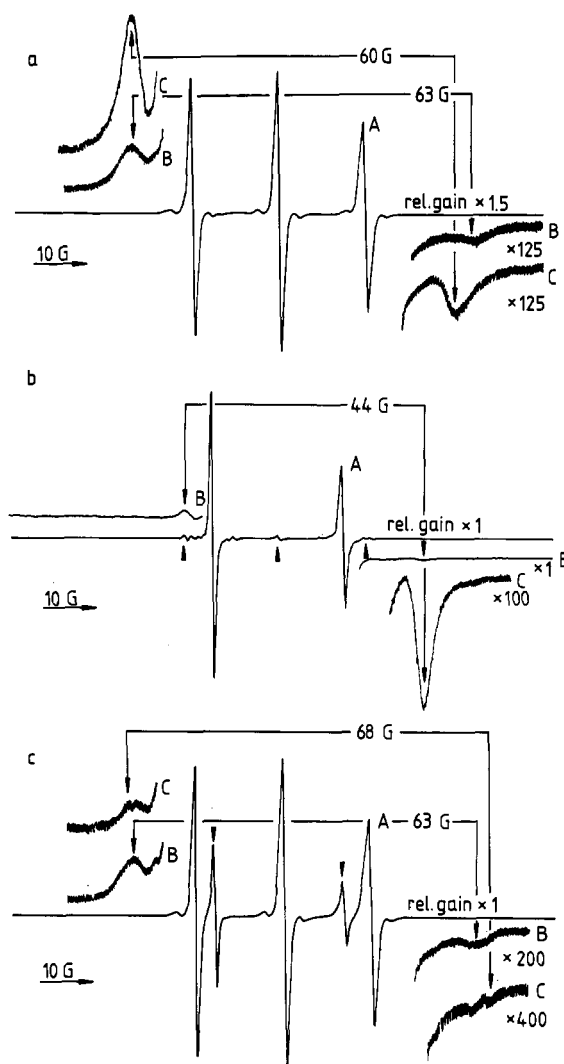


FIGURE 8: (Part a) ESR spectra of 180 μM AcAc-pantetheine-SL at pH 7.2 in Tris-HCl buffer in the absence (trace A) and in the presence (traces B and C) of 350 μM L-3-hydroxyacyl-CoA dehydrogenase. Trace C: After addition of 420 μM N^6 -SL- NAD^+ . (Part b) ESR spectra of 340 μM N^6 - ^{15}N , $^2\text{H}_{17}$]SL- NAD^+ at pH 6.8 in pyrophosphate buffer in the absence (trace A) and in the presence (trace B) of 340 μM L-3-hydroxyacyl-CoA dehydrogenase. The upward arrows in spectrum A indicate an about 1% ^{14}N impurity in N^6 - ^{15}N , $^2\text{H}_{17}$]SL- NAD^+ . (Part c) ESR spectra of AcAc-pantetheine-SL and N^6 - ^{15}N , $^2\text{H}_{17}$]SL- NAD^+ as in part b. Trace B: 300 μM AcAc-pantetheine-SL in the presence of 250 μM L-3-hydroxybutyrate dehydrogenase. Traces A and C: after addition of 350 μM N^6 - ^{15}N , $^2\text{H}_{17}$]SL- NAD^+ . The downward arrows in part c indicate ^{15}N signals.

and the spin-labeled substrates exhibit maximum velocities comparable to those of the native compounds, i.e., varying from 20% to 70%. Likewise, the equilibrium binding constants are hardly changed at all by the presence of the label.

In Figure 9 are shown molecular models of the active centers within the dimeric structure of L-3-hydroxyacyl-CoA dehydrogenase. The spin-labeled substrates AcAc-pantetheine-SL and AcAc-cysteamine-SL have been fitted into the active clefts as proposed by Birktoft et al. (1987) for the "more favorable orientation" (Figure 1). Each subunit of the enzyme is composed of two domains. The fatty acid moiety of the substrates has been placed in the cleft between the domains so as to allow for hydride transfer between C4 of the nicotinamide ring and the β -carbonyl group of the fatty acid.

By comparison of AcAc-pantetheine-SL (Figure 9, top) and AcAc-cysteamine-SL (Figure 9, bottom), it is immediately obvious that the spin-labels of two AcAc-pantetheine-SL

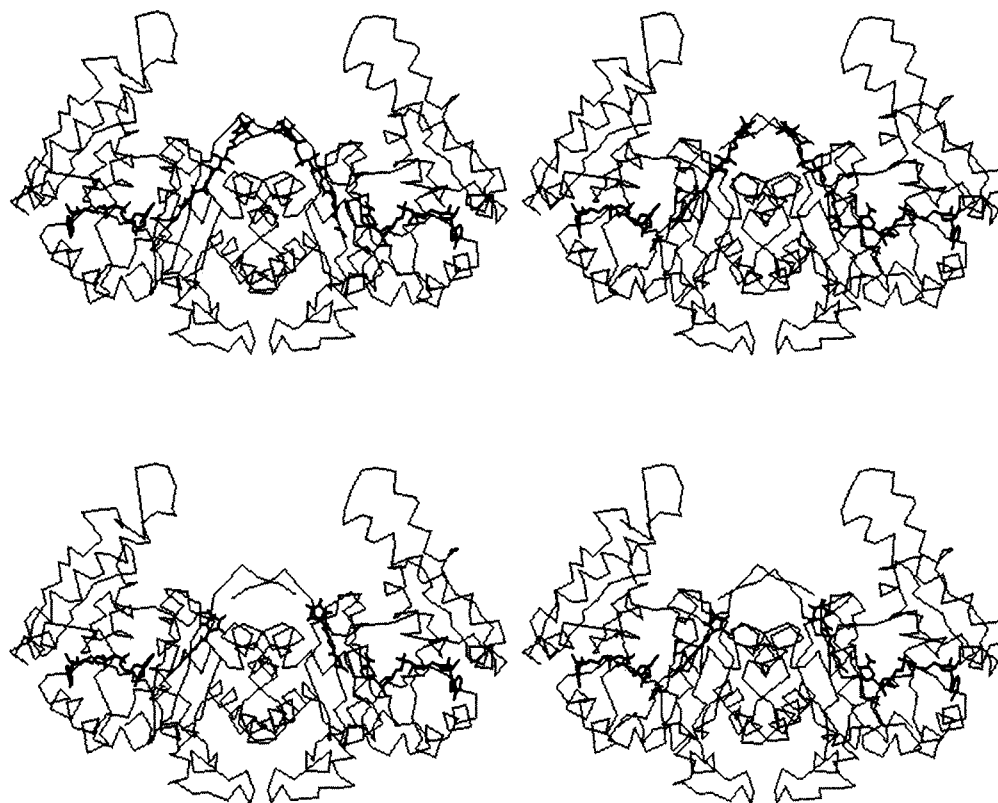


FIGURE 9: Top: Stereo diagram of a molecular model of the hypothetical ternary complex of the L-3-hydroxyacyl-CoA dehydrogenase dimer ($C\alpha$ atoms, dotted line) with coenzyme NAD^+ and AcAc-pantetheine-SL (solid lines). The substrate analogue is fitted with the fatty acid moiety into the cleft between the domains of each subunit in such a way as to allow for hydride transfer to C4 of the nicotinamide ring. The distance between the two nitroxide residues is 6.5 Å and varies from 4.0 to 10.0 Å upon rotation about the ester bond between the spin-label and AcAc-pantetheine as well as the bond between the carbonyl carbon of the spin-label and its pyrroline ring. Bottom: As in part a but with AcAc-cysteamine-SL as substrate. The distance between the two nitroxide residues is 16 Å and varies from 14 to 20 Å depending upon rotation about the amide bond between the spin-label and AcAc-cysteamine as well as the bond between the carbonyl carbon of the spin-label and its pyrroline ring.

molecules are very close to one another (about 4 Å between the nitroxide groups) upon binding to both active sites within the dimer. In the case of AcAc-cysteamine-SL, the minimum distance is larger by about 10 Å. This finding is in excellent agreement with our ESR data. At molar excesses over enzyme that allow for considerable occupancy of both sites within the dimer, AcAc-pantetheine-SL showed strong spin-spin interaction corresponding to a distance of 8.5–12 Å as compared to 4–10 Å in the molecular model. Under comparable conditions, AcAc-cysteamine-SL exhibited an interaction that could result from nitroxides that are separated by about 13–21 Å compared to 14–20 Å in the molecular model. Table II summarizes these distances.

In the alternative arrangement of the substrates (Birktoft et al., 1987), i.e., with the CoA moiety located in the cleft between the two domains, the distance between the nitroxide groups of two substrates bound to different subunits within the dimer is too large for spin-spin interaction to be directly observable. Estimates from the crystal coordinates suggest that the separation would be more than 30 Å for AcAc-cysteamine-SL and even more than 40 Å in the case of AcAc-pantetheine-SL.

The ESR spectra of AcAc-pantetheine-SL and AcAc-cysteamine-SL at low saturation fractions, i.e., in the absence of spin-spin interaction, lend further support to our model. In the case of AcAc-pantetheine-SL (Figure 9a), the spin-label is motionally less restricted according to the molecular model as well as the ESR data. The A_{zz} value of 62–63 G as compared 65 G for AcAc-cysteamine-SL indicates higher mobility. The separation of the outer extrema generally depends (in the

Table II: Comparison of Nitroxide-Nitroxide Distances between Spin-Labeled Substrates Bound to the L-2-Hydroxyacyl-CoA Dehydrogenase Dimer As Derived from ESR Spectroscopy^a and Molecular Modeling

compound	max splitting (G)	distance in Å as obtained by	
		ESR simulation	molecular modeling
AcAc-pantetheine-SL	105	8.5–12	4–10
AcAc-cysteamine-SL	78	13–21	14–20

^a Distances were determined by complete spectral simulation as described in the text. In column 2 the maximum splitting of the outer extrema arising from spin-spin interaction is given.

absence of spin-spin interaction) on motion of the spin-label as well as, to a lesser extent, polarity in its vicinity (Griffith & Waggoner, 1969).

When both spin-labeled substrate and spin-labeled NAD^+ were used, changes in the ESR spectrum of bound AcAc-pantetheine-SL were observed upon addition of N^6 -[^{15}N , $^2H_{17}$]SL- NAD^+ . Two new maxima formed, one each in the low-field and the high-field regions. The relatively small separation of 68 G between the outermost bands could arise from dipolar splitting, but may simply reveal two distinct environments of the substrate in the presence of the coenzyme. As pointed out above, the separation generally depends on motion of the spin-label as well as on polarity in its vicinity (Griffith & Waggoner, 1969). For spin-spin interaction between N^6 -[^{15}N , $^2H_{17}$]SL- NAD^+ and AcAc-pantetheine-SL to be detectable, the nitroxide-nitroxide distance must be ≤ 20 Å. This is only possible when the substrate is unfavorably

bound with the CoA moiety located in the cleft between the two domains, resulting in a distance between the nitroxide groups of about 15–20 Å as compared to more than 30 Å in the reverse orientation. However, this arrangement can be excluded because the large peak separation of 105 G observed with AcAc-pantetheine-SL alone under saturating conditions must result from spin–spin interaction, which is restricted to an arrangement with the fatty acid chain placed in the cleft. That, indeed, the additional bands are not due to dipolar interaction between AcAc-pantetheine-SL and N^6 -[^{15}N , $^2\text{H}_{17}$]SL-NAD $^+$ was shown unequivocally by the addition of unlabeled NAD $^+$, which had the same effect. Hence, the coenzyme induces conformational changes in the binary enzyme/substrate complex that are monitored by the spin-label of AcAc-pantetheine-SL.

A puzzling result has been the lack of ESR signals from bound N^6 -SL-3'-dephospho-AcAc-CoA in the presence of L-3-hydroxyacyl-CoA dehydrogenase. Binding of N^6 -SL-3'-dephospho-AcAc-CoA in the active site was established by the reversible decrease of the signal amplitude of the free analogue in the presence of enzyme (upon addition of AcAc-CoA) and by its enzymatic turnover. A similar finding was described by Streckenbach et al. (1980) for a spin-labeled ATP binding to (Na $^+$, K $^+$)ATPase and was attributed to a very high degree of motional freedom of the spin-label in spite of the complex formation of the ATP moiety of the analogue with the enzyme. On the basis of our model for substrate binding to L-3-hydroxyacyl-CoA dehydrogenase as shown in Figure 9a for AcAc-pantetheine-SL, the spin-label in the considerably longer N^6 -SL-3'-dephospho-AcAc-CoA should indeed be highly mobile. On the other hand, it is hard to imagine that there should be not the slightest broadening of the triplet from free N^6 -SL-3'-dephospho-AcAc-CoA. An alternative explanation for the lack of a bound signal is given by the extremely close proximity of the two nitroxide groups that would seem possible within the dimer. Hence, the bound signal could be broadened out completely.

In conclusion, our ESR data show that binding of CoA derivatives of fatty acids to L-3-hydroxyacyl-CoA dehydrogenase occurs in such a way that the hydrocarbon chain interacts with a portion of the enzyme. In contrast, a large part of the CoA moiety is motionally free. As judged by the spin-label studies, it does not seem to interact with the enzyme, and we suggest that it does not contribute to either binding energy or binding specificity. This agrees with earlier biochemical observations in which substrate analogues of fatty acyl CoA react equally well with and without most of the CoA moiety. The spin-label studies also aid in analyzing modes of substrate binding on the basis of the crystal structure of the enzyme with and without the coenzyme NAD $^+$. In the X-ray studies, no experimental data could be obtained on the binding of the fatty acid substrates. However, even without a complete molecular model, the active site was determined by studying the location of bound NAD $^+$. When computer graphics are used to analyze alternate binding modes of the fatty acyl CoA substrate to the crystal structure, many positions are possible. However, when the C3 of the substrate is positioned near the 4-position of the nicotinamide ring of the coenzyme, binding modes were far more limited. Such stereochemical proximity is an accepted form of intermediate for direct hydride transfer during a dehydrogenase reaction. The modeling studies with L-3-hydroxyacyl CoA dehydrogenase suggested two most probable orientations. In one, the fatty acyl chain of the substrate lies in a cleft separating the two domains of a single subunit. In this preferred hypo-

thetical location, the CoA end of the substrate in the two subunits of the enzyme can be relatively close to each other. This agrees with the spin-labeling results described above. The model derived from the ESR studies and the crystallographic model is attractive because the length of the hydrocarbon portion would be of secondary importance to binding and turnover and much of the CoA moiety would not contribute to binding specificity. Both are known biochemical properties of this dehydrogenase. It must be pointed out that in this investigation the shortest possible fatty acid chain of four carbon atoms was employed, only. However, given the potential additional hydrophobic interactions of a longer chain with the enzyme when located in the cleft, the same arrangement of the substrate is to be expected.

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A Kinetic Study of the Competition between Renaturation and Aggregation during the Refolding of Denatured-Reduced Egg White Lysozyme[†]

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ABSTRACT: The recovery of proteins following denaturation is optimal at low protein concentrations. The decrease in yield at high concentrations has been explained by the kinetic competition of folding and "wrong aggregation". In the present study, the renaturation-reoxidation of hen and turkey egg white lysozyme was used as a model system to analyze the committed step in aggregate formation. The yield of renatured protein for both enzymes decreased with increasing concentration in the folding process. In addition, the yield decreased with increasing concentrations of the enzyme in the denatured state (i.e., prior to its dilution in the renaturation buffer). The kinetics of renaturation of turkey lysozyme were shown to be very similar to those of hen lysozyme, with a half-time of about 4.5 min at 20 °C. The rate of formation of molecular species that lead to formation of aggregates (and therefore fail to renature) was shown to be rapid. Most of the reaction occurred in less than 5 s after the transfer to renaturation buffer, and after 1 min, the reaction was essentially completed. Yet, by observing the effects of the delayed addition of denatured *hen* lysozyme to refolding *turkey* lysozyme, it was shown that folding intermediates become resistant to aggregation only much more slowly, with kinetics indistinguishable from those observed for the appearance of native molecules. The interactions leading to the formation of aggregates were nonspecific and do not involve disulfide bonds. These observations are discussed in terms of possible kinetic and structural aspects of the folding pathway.

Refolding denatured polypeptide chains in vitro has been an important issue for both basic research and applied biotechnology. Indeed, for several decades, the major advances toward the understanding of protein folding [the "second

translation of the genetic message" (Goldberg, 1985)] have come from studies of the renaturation of proteins denatured by urea, guanidine, pH, or heat. Recently, with the advent of recombinant DNA technology, it has become simple to express a gene of interest in a foreign cell and to obtain large amounts of the desired polypeptide chain. However, such polypeptide chains are often produced as insoluble, biologically inactive "inclusion bodies" (Marston, 1986; Jaenicke, 1987). To obtain the functional protein from the inclusion bodies, it is usually necessary to dissolve the inclusion bodies in a denaturing medium and to submit the solubilized protein to a renaturation procedure (Kane & Hartley, 1988; Rudolph, 1990). Ideally, renaturation procedures in basic research and

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