Distribution of Reaction Intermediates on Chicken Liver Fatty Acid Synthase†

Vernon E. Anderson‡ and Gordon G. Hammes*
Department of Chemistry, Cornell University, Ithaca, New York 14853
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ABSTRACT: The distributions of covalent intermediates in the reaction cycle catalyzed by chicken liver fatty acid synthase were studied. In isotope-trapping experiments, 30% of [1-^{14}C]acetyl-enzyme and 6.7% of [2-^{14}C]malonyl-enzyme are converted to long-chain fatty acids, indicating the initiation reaction is partially random. The 3-hydroxybutyryl intermediate is located on the tryptic peptide which contains the 4′-phosphopantetheine (>90%), while the C_4–C_{18} saturated intermediates are distributed both on this peptide and on the peptide that contains the active cysteine. The ratio of intermediates on the two peptides is about unity for chain lengths $<C_{14}$, but the amount on the active cysteine progressively decreases for chain lengths of C_{14}, C_{16}, and C_{18} with trypsinized enzyme. The distributions of carbon chain lengths for the saturated or 3-keto intermediates when acetoacetyl-labeled trypsinized enzyme is incubated with limiting malonyl coenzyme A or NADPH, respectively, show large fractions both of unreacted enzyme and of C_{16} or longer intermediates. A detailed analysis suggests that the initial condensation and reduction steps are slower than the analogous reactions with longer chain length intermediates. The 3-keto intermediate comprises over 70% of each chain length intermediate detected when NADPH is the limiting substrate, indicating the reduction of the 3-keto intermediates is at least 2 times slower than the reduction of the unsaturated intermediates.

Fatty acid synthase is a multifunctional enzyme that catalyzes the synthesis of long-chain fatty acids according to the overall reaction

$$\text{acetyl-CoA} + n\text{malonyl-CoA} + 2n\text{NADPH} + 2n\text{H}^+ \rightarrow \text{CH}_3(\text{CH}_2\text{CH}_2)_n\text{COOH} + (n+1)\text{CoA} + 2n\text{NADP}^+ + (n-1)\text{H}_2\text{O} + n\text{CO}_2 \quad (1)$$

This is a controlled polymerization reaction in which the intermediates are sequestered by thio ester linkages to the enzyme [cf. Lynen (1967) and Wakil et al. (1983)]. Polymerization reactions can be divided into initiation, elongation, and termination reactions. The fatty acid synthase initiation reaction requires the transfer of an acetyl and a malonyl moiety from their coenzyme A (CoA)[1] thio esters to an active cysteine and the thiol of the 4′-phosphopantetheine prosthetic group, respectively (Joshi et al., 1970; Phillips et al., 1970). This initiation reaction has been suggested to be an ordered addition of substrates (Soulié et al., 1983; McCarthy et al., 1983). During the elongation reaction, a saturated acyl intermediate on the active cysteine is condensed with a malonyl moiety bound to the 4′-phosphopantetheine. The resulting 3-keto intermediate is reduced to an alcohol, dehydrated, and reduced, ultimately resulting in another saturated acyl intermediate linked to the active cysteine. This requires that the intermediate be transferred from the 4′-phosphopantetheine to the active cysteine, presumably after the final reduction.

The locations of the reaction intermediates on the enzyme are investigated in this study by specifically radioactively labeling the intermediates and separating the 4′-phosphopantetheine intermediates (peptide II) from the active cysteine intermediates (peptide I) by limited trypsinization (Mattick et al., 1983). The relative rates of the condensation and reduction reactions as a function of chain length are determined from the relative concentrations of different carbon chain intermediates produced when the enzyme is incubated with limiting malonyl-CoA or NADPH, respectively.

Materials and Methods

Enzymes. Fatty acid synthase was prepared according to Cognet & Hammes (1983) with omission of the final agarose column. The enzyme had a specific activity of 1.5–1.6 units/mg under standard conditions (Cardon & Hammes, 1982). Trypsin treated with L-1-(tosylamido)-2-phenylethyl chloromethyl ketone was from Worthington Biochemicals. All other enzymes and CoA esters were from Sigma. [1-^{3}H]Glucose was from ICN, [1-^{14}C]acetyl-CoA was from Amersham; and [2-^{14}C]malonyl-CoA was from New England Nuclear.

(S)-[4-^{3}H]NADPH. $NADP^+$ was reduced by [1-^{3}H]glucose 6-phosphate in the presence of glucose-6-phosphate dehydrogenase, and the product (*S*)-[4-^{3}H]NADPH (1100 cpm/nmol) was purified as described by Anderson & Hammes (1984).

(R)-[4-^{3}H]NADPH. Five milliliters of ca. 1 μM (*S*)-[4-^{3}H]NADPH in 150 mM sodium phosphate, pH 7.3, was oxidized to [4-^{3}H]$NADP^+$ by adding 50 μmol of sodium pyruvate, 5 μmol of magnesium acetate, 0.2 μmol of Na_2EDTA, and 1 unit of malic enzyme. The disappearance of the NADPH fluorescence (excitation 340 nm, emission 465 nm) was followed for 10 half-lives and the reaction terminated by adding an additional 10 μmol of Na_2EDTA. The [4-^{3}H]-$NADP^+$ was reduced to (*R*)-[4-^{3}H]NADPH in situ by the addition of 0.3 μmol of glucose 6-phosphate and 0.2 unit of glucose-6-phosphate dehydrogenase. The reaction mixture was diluted to 20 mL prior to loading it on a 3-mL DEAE-Sephacel column preequilibrated with 20 mM disodium phosphate. The column was eluted with a 100-mL linear gradient from 0.02 to 0.2 M disodium phosphate. The stereochemical purity was better than 90% as determined by the reduction of α-ketoglutarate and ammonia in the presence of glutamate de-

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[1] Abbreviations: CoA, coenzyme A; EDTA, ethylenediaminetetraacetic acid.

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hydrogenase (Anderson & Hammes, 1984).

Separation of Peptides Containing 4'-Phosphopantetheine and the Active Cysteine. Peptides containing the active cysteine and 4'-phosphopantetheine were separated by subjecting the enzyme to a limited trypsinization followed by polyacrylamide gel electrophoresis. This procedure separates peptide I (M_r = 127 000) containing the active cysteine from peptide II (M_r = 107 000) containing the 4'-phosphopantetheine (Mattick et al., 1983). Trypsin was added to concentrated fatty acid synthase (1:1000 by weight) in 0.1 M potassium phosphate, pH 7.0, 1 mM EDTA, 10 mM dithiothreitol, and 10% glycerol. The proteolysis was monitored by removing small aliquots of enzyme and determining the decrease in activity. When less than 2% of the initial activity remained, the proteolysis was stopped by adding a 3-fold weight excess, relative to trypsin, of soybean trypsin inhibitor. After being labeled with radioactive substrates, the enzyme was electrophoresed on sodium dodecyl sulfate [0.4% bis(acrylamide)]–8% polyacrylamide gels (Laemmli, 1970). To provide the greatest resolution of peptide I and peptide II, the electrophoresis was carried out for 50% longer than required for the bromthymol blue tracking dye to pass through the gel. The three major bands of M_r 220 000, 127 000, and 107 000 were well separated after staining with Coomassie blue and destaining with 20% ethanol and 10% acetic acid.

Analysis of Polyacrylamide Gels. The destained polyacrylamide gels were divided into five slices containing one of the peptides or the blank space between the peptides. The amount of radioactivity in the slices was determined either by dissolving the gel in 0.5 mL of 30% hydrogen peroxide at 90 °C for 2 h followed by the addition of 200 units of catalase to destroy the excess peroxide or by converting the radioactive thio ester intermediates to benzylamides by incubating the gel slices in 400 μL of 10% benzylamine for 12 h on a gel rocker. The benzylamine solution was removed from the gel slice and added to 1.0 mL of 0.4 M monosodium phosphate. The gel slices were extracted twice more for 4 h, once with 1.0 mL of water and once with 1.0 mL of 2-propanol. Both the water and 2-propanol extracts were added to the neutralized benzylamine solution, and the benzylamides were obtained from this solution by extracting 3 times with 3.0 mL of chloroform. The combined chloroform extracts were extracted once with 1.0 mL of 0.2 M monosodium phosphate, twice with 1.0 mL of water, and then evaporated to dryness under a stream of dry nitrogen.

Liquid Chromatography Analysis of Benzylamides. 3-Hydroxybutyrylbenzylamide, crotonylbenzylamide, and butyrylbenzylamide were separated by chromatography on a reverse-phase C_{18} column as described previously (Anderson & Hammes, 1984). The benzylamides of the saturated intermediates, from C_4 through C_{20}, were separated with a linear 60–95% (CH_3OH/H_2O) gradient on a 4.6 mm × 25 mm Econosphere C-18 column (Alltech) as shown in Figure 1. The standard benzylamides were synthesized from either their acid chloride or their anhydride derivatives. For both chromatographic procedures, 1.0-mL fractions were collected and added directly to 10 mL of aqueous counting solution (Amersham) and counted in a Beckman LS-200C scintillation counter.

Location of C_4 Intermediates. Trypsinized fatty acid synthase, typically 100 μL of 1 μM enzyme, was incubated with a combination of priming substrates (300 μM acetoacetyl-CoA, 300 μM crotonyl-CoA, or 100 μM acetyl-CoA and 50 μM malonyl-CoA) and varying concentrations of either (*R*)- or (*S*)-[4-^{3}H]NADPH. These reactions were quenched after 15 s by the addition of 30 μL of an aqueous solution containing 10% acetic acid, 10% glycerol, and 2% sodium dodecyl sulfate. These samples were transferred directly to the wells of a polyacrylamide gel which was electrophoresed and analyzed as described above.

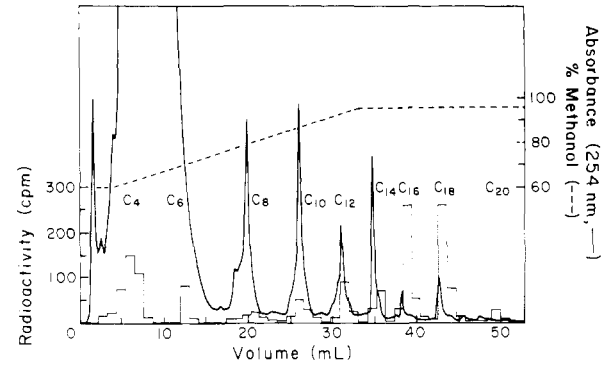

FIGURE 1: Reverse-phase high-performance liquid chromatography of a mixture of standard saturated fatty acid benzylamides and radioactive intermediates extracted from a polyacrylamide gel slice containing peptide II: absorbance at 254 nm (—); radioactivity contained in 1-mL fractions (bars); percent methanol in the water/methanol gradient (---). The carbon chain lengths were assigned by comparison of elution volumes with single standards.

Determination of the Transesterification Equilibrium Constant. Trypsinized fatty acid synthase, final concentration 1 μM, was added to a solution of 100 μM acetoacetyl-CoA, 15 μM (*S*)-[4-^{3}H]NADPH, and varying concentrations of malonyl-CoA. After 30 s, the reaction was quenched by the addition of 30 μL of an aqueous solution containing 10% acetic aicd, 10% glycerol, and 2% sodium dodecyl sulfate. The quenched reaction mixture was electrophoresed on a polyacrylamide gel, and the benzylamide derivatives of the radioactive intermediates from peptide I and from peptide II were isolated and chromatographed as described above. The equilibrium constant for each chain length with an even number of carbons was taken to be the ratio of counts purified from peptide I and peptide II for each respective chain length.

Relative Abundance of Intermediates with Limiting Malonyl-CoA. The relative abundance of the saturated intermediates formed in the presence of limiting malonyl-CoA was determined by incubating 5 μM fatty acid synthase, either native or trypsinized to 1–2% of the initial activity, with 300 μM acetoacetyl-CoA for 15 s, after which the excess acetoacetyl-CoA was removed by passing the solution through a 3-mL Sephadex G-50 fine centrifuge column preequilibrated with 100 mM sodium phosphate, pH 7.0, and 1 mM EDTA. Two hundred forty microliters of the effluent was added to 360 μL of a solution containing 6 μM malonyl-CoA and 25 μM (*S*)-[4-^{3}H]NADPH. The reactions were quenched by the addition of 50 μL of acetic acid at various times. The excess radioactive label was removed by dividing the quenched reaction mixture into two 325-μL aliquots and passing them each through a 3-mL Sephadex G-50 fine centrifuge column preequilibrated with 50 mM potassium acetate, pH 4.0. The effluents were pooled, and the covalent intermediates were converted to their benzylamides and isolated as described above. To correct for the varying number of tritium atoms incorporated, the radioactivity eluting with the benzylamide of carbon chain length n was divided by $n/2 - 1$.

Relative Abundance of Intermediates with Limiting NADPH. Figure 2 illustrates the protocol used to determine the relative amounts of the unreduced intermediates present on fatty acid synthase when the enzyme, primed with acetoacetyl-CoA, is reacted with an excess of malonyl-CoA and less

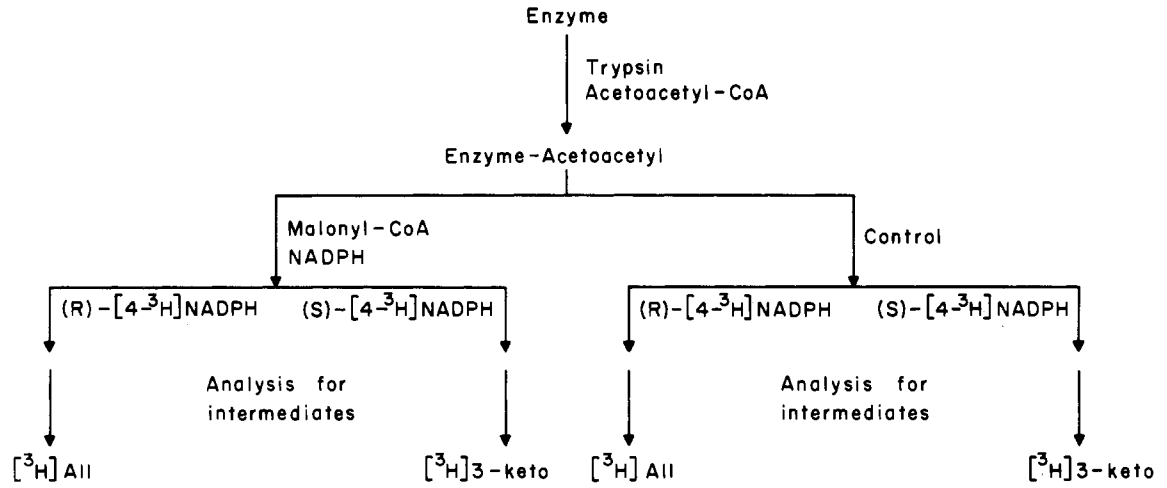

FIGURE 2: Experimental scheme for determining the relative abundance of intermediates formed in the presence of limiting NADPH. Conditions for each step are given under Materials and Methods.

than stoichiometric amounts of NADPH. Trypsinized enzyme, 1.8 mL of 5.0 μM, was incubated with 100 μM acetoacetyl-CoA. After 15 s, the excess acetoacetyl-CoA was removed by passing 300-μL aliquots through six separate 3-mL Sephadex G-50 fine centrifuge columns (Penefsky, 1977) preequilibrated with 100 mM sodium phosphate, pH 7.0, and 1 mM EDTA and centrifuged for only 1 min. The eluates were pooled and were typically 1.7 mL of ca. 2.5 μM acetoacetyl-labeled and trypsinized fatty acid synthase. This enzyme (240 μL) was added to 360 μL of solution containing 83 μM malonyl-CoA and either 3.3 or 13.3 μM NADPH in 0.1 M sodium phosphate, pH 7.0. Excess malonyl-CoA and $NADP^+$ were removed by passing 300-μL aliquots through 3-mL centrifuge columns as above. The eluates from the two columns were pooled, and 250-μL aliquots were added to 750 μL containing 2 μM either of (*R*)- or of (*S*)-[4-^{3}H]NADPH. After 15 s, these reactions were quenched by the addition of 1.0 mL of 8 M guanidine hydrochloride. These samples were then chromatographed on a 10-mL Sephadex G-25 fine column run at 0.7 mL/min with 4 M guanidine hydrochloride as eluant. The fractions containing protein (determined by the absorbance at 280 nm) were pooled, and the thio ester intermediates were converted to their benzylamide derivatives by adding benzylamine to 0.4 M and monosodium phosphate to 0.2 M. After 3 h, the benzylamides were extracted with chloroform and chromatographed on a C_{18} reverse-phase column as described above. This procedure produces two sets of saturated intermediates, one labeled with (*R*)-[4-^{3}H]-NADPH, which labels the 3-keto, 3-hydroxy, and 2,3-unsaturated intermediates, and the other labeled with (*S*)-[4-^{3}H]NADPH, which labels only the 3-keto intermediates. The ratio of radioactivity obtained from the (*S*)- to the (*R*)-[4-^{3}H]NADPH-labeled samples gives an estimate of the fraction of each chain length intermediate that is present as the 3-keto intermediate. To determine the value of this ratio for a known intermediate, 100 μL of the pooled acetoacetyl-labeled and trypsinized fatty acid synthase was added to 1.0 mL of either 2 μM (*R*)-[4-^{3}H]NADPH or 2 μM (*S*)-[4-^{3}H]NADPH. After 15 s, 1.0 mL of 8 M guanidine hydrochloride was added and the sample chromatographed on the 10-mL Sephadex G-25 fine column as described above. The specific activity of each sample was measured by determining the absorbance at 280 nm and the radioactivity in 100 μL of the peak protein fraction. The ratio of the specific activities of the (*S*)- to (*R*)-[4-^{3}H]NADPH-labeled samples gives the value of this ratio when the fraction of 3-keto intermediate is known to be 1.0. Conversion of the (*S*)-[4-^{3}H]NADPH-labeled sample to the benzylamide derivatives and chromatography as described above confirmed that the acetoacetyl intermediate was fully reduced.

Conversion of [1-^{14}C]Acetyl- and [2-^{14}C]Malonyl-Labeled Enzyme to Product. Two micromolar fatty acid synthase was incubated in 300 μL of 10 μM [2-^{14}C]malonyl-CoA or 10 μM [1-^{14}C]acetyl-CoA in 100 mM sodium phosphate, pH 7.0, for 15 s. The solutions were passed through a 3-mL Sephadex G-50 fine column preequilibrated with 100 mM sodium phosphate, pH 7.0, to remove the excess radioactive CoA esters. Fifty-microliter aliquots of the [2-^{14}C]malonyl-labeled enzyme [0.8–1.0 [2-^{14}C]malonyl/enzyme (mol/mol)] or the [1-^{14}C]acetyl-labeled enzyme [1.8 [1-^{14}C]acetyl/enzyme (mol/mol)] were then added to 2.0 mL containing 10 μM NADPH, 100 μM acetyl-CoA, and 200 μM malonyl-CoA. After 30 s, the reactions were quenched by the addition of 50 μL of acetic acid. The reaction mixture was extracted 6 times with 2.0 mL of cyclohexane; the first three and last three extracts were pooled separately. Both cyclohexane pools were extracted twice with 1.0 mL of distilled water. The two cyclohexane extract pools, the two aqueous extract pools, and the remaining reaction solution were each added to 14 mL of aqueous counting scintillation fluid, and the radioactivity was determined on the broad ^{3}H + ^{14}C channel of the Beckman LS 200C scintillation counter. The sum of the counts in the cyclohexane pools results from [2-^{14}C]malonyl- or [1-^{14}C]-acetyl-labeled enzyme that has been converted to product, i.e., long-chain fatty acids. The sum of the counts in the three aqueous solutions results from [2-^{14}C]malonyl- or [1-^{14}C]-acetyl-labeled enzyme that was not converted to product.

Spectroscopic Measurements. The following extinction coefficients were used to determine concentrations: fatty acid synthase, $\epsilon_{280} = 4.82 \times 10^5$ M^{-1} cm^{-1} (Hsu & Yun, 1970); CoA thio esters, $\epsilon_{260} = 1.54 \times 10^4$ M^{-1} cm^{-1}; NADPH, $\epsilon_{340} = 6220$ M^{-1} cm^{-1} (P-L Biochemicals, circular OR-10).

Numerical Solutions of Differential Equations. The simultaneous differential equations generated by the models described under Discussion were solved with a Fortran computer program using the DGEAR subroutine of the International Mathematics and Statistics Library.

RESULTS

Location of C_4 Intermediates. The location of the 3-hydroxybutyryl and butyryl intermediates was determined first by varying the reaction conditions so that the radioactive

Table I: Location of Four-Carbon Intermediates[a]

substrate	[substrate] (μM)	stereospecificity of [4-^{3}H]NADPH	[4-^{3}H]NADPH concn (μM)	radioactivity (cpm) peptide I[b]	peptide II[b]	peptide I/ peptide II
acetoacetyl-CoA	300	*R*	0.3 (480 cpm/pmol)	254	183	1.4
acetoacetyl-CoA	300	*S*	0.3 (1100 cpm/pmol)	1229	3035	0.4
crotonyl-CoA	300	*R*	0.3 (480 cpm/pmol)	1350	800	1.7
malonyl-CoA	100	*R*	50 (3 cpm/pmol)	20	407	0.05
acetyl-CoA	50					
acetyl-CoA	50	*S*	50 (6 cpm/pmol)	53	655	0.08

[a] 1 μM trypsinized enzyme, 0.1 M sodium phosphate, pH 7.0, and 1 mM EDTA. [b] Gel slices were dissolved in 30% H_2O_2 as described under Materials and Methods.

Table II: Location of Four-Carbon Intermediates[a]

substrate	[substrate] (μM)	[4-^{3}H]NADPH stereospecificity	[4-^{3}H]NADPH concn (μM)	peptide[b]	3-hydroxybutyryl (cpm)	butyryl (cpm)
acetoacetyl-CoA	300	*R*	0.3	I	<10	102
acetoacetyl-CoA	300	*R*	0.3	II	<10	64
crotonyl-CoA	300	*R*	0.3	I	<10	460
crotonyl-CoA	300	*R*	0.3	II	<10	191
acetoacetyl-CoA	300	*S*	0.3	I	124	256
acetoacetyl-CoA	300	*S*	0.3	II	1202	99
acetoacetyl-CoA	300	*S*	10	I	<20	1118
acetoacetyl-CoA	300	*S*	10	II	<20	712
acetyl-CoA	100	*S*	0.1	I	100	78
malonyl-CoA	50					
malonyl-CoA	50	*S*	0.1	II	499	37

[a] 1 μM trypsinized enzyme, 0.1 M sodium phosphate, pH 7.0, and 1 mM EDTA. [b] Gel slices were derivatized with benzylamine as described under Materials and Methods.

Table III: Distribution of Intermediates with Limiting Malonyl-CoA[a]

carbon chain length	malonyl-CoA concn (μM) 0.33 peptide I (cpm)	peptide II (cpm)	I/II ratio	% of total intermediates	1.0 peptide I (cpm)	peptide II (cpm)	I/II ratio	% of total intermediates
4	450	230	2	57	160	95	2	42
6	180	85	2	11	55	20	3	6
8	195	50	4	7	70	25	3	5
10	170	100	2	6	80	60	1	6
12	200	115	2	5	100	75	1	6
14	180	130	1	4	50	55	0.9	3
16	110	340	0.3	5	70	180	0.4	6
18	<20	345	<0.06	4	50	1055	0.05	22
20	<20	35	<0.6	0.3	<20	235	<0.08	4

[a] 1 μM trypsinized enzyme, 20 μM (*S*)-[4-^{3}H]NADPH, 100 μM acetoacetyl-CoA, 0.1 M sodium phosphate, pH 7.0, 1 mM EDTA, and 0.33 or 1.0 μM malonyl-CoA.

intermediate would be predominantly either 3-hydroxybutyryl or butyryl (Cognet & Hammes, 1985). Since the 3-keto reductase transfers the *pro-S* hydrogen and the enoyl reductase transfers the *pro-R* hydrogen of NADPH (Anderson & Hammes, 1984; Dugan et al., 1970), only the butyryl intermediates are labeled by (*R*)-[4-^{3}H]NADPH. The first and third rows in Table I indicate that the butyryl intermediate is found on both peptide I, which contains the active cysteine, and peptide II, which contains the 4′-phosphopantetheine, in a ratio of 1.4 to 1.7. The 3-hydroxybutyryl intermediate was labeled by limiting the ratio (*S*)-[4-^{3}H]NADPH/acetoacetyl-enzyme to less than 0.33. This intermediate is found predominantly on peptide II (row 2, Table I). The ratio of 3-hydroxybutyryl on the two peptides cannot be accurately determined from these data because some of the radioactivity is present as the butyryl intermediate. Rows 4 and 5 indicate that the trypsinized enzyme is capable of incorporating several equivalents of [4-^{3}H]NADPH into intermediates; i.e., the trypsinized enzyme is capable of performing all of the catalytic reactions to synthesize long-chain fatty acids except the hydrolysis of the final product. These long-chain intermediates were found almost solely on peptide II.

Table II presents the results of the chromatography which separates the four-carbon intermediates obtained from peptide I and peptide II. Rows 1–4 verify that only the butyryl intermediate was labeled by incubation with (*R*)-[4-^{3}H]-NADPH. Rows 5 and 6 indicate that about 90% of the 3-hydroxybutyryl derivative is found on peptide II, in contrast to the average of 30% for the butyryl derivative. The data in rows 7 and 8 were obtained with excess NADPH and indicate the intermediates were quantitatively reduced to butyryl. The data in rows 9 and 10 were obtained with acetyl-CoA, malonyl-CoA, and a limiting amount of NADPH; the results are similar to those obtained with acetoacetyl-CoA as the primer (rows 5 and 6). No radioactivity coeluted with carrier crotonylbenzylamide. Either the crotonyl-enzyme is present in small amounts or it is not derivatized by benzylamine. This question is addressed later.

Location and Relative Abundance of Intermediates with Limiting Malonyl-CoA. Figure 1 is a chromatograph obtained with the benzylamides extracted from peptide II when the enzyme was reacted with 0.33 μM malonyl-CoA (column 2, Table III). The superimposed histogram of the radioactivity indicates that all of the radioactivity coelutes with a standard

Table IV: Relative Abundance of Intermediates (%)[a]

carbon chain length	time (s) 15[b]	15[c]	120[b]	120[c]
4	58	61	36	56
6	7	16	11	8
8	4	11	7	7
10	6	8	9	8
12	6	3	9	8
14	3	1.3	6	11
16	8	0.1	14	1
18	6	<0.1	7	<0.1
20	0.3	<0.1	0.5	<0.1

[a] Approximately 1 μM acetoacetyl-enzyme, 3.6 μM malonyl-CoA, 25 μM (*S*)-[4-^{3}H]NADPH, 0.1 M sodium phosphate, pH 7.0, and 1 mM EDTA. [b] Trypsinized enzyme. [c] Native enzyme.

fatty acyl benzylamide of specified chain length. The butyryl- and hexanoylbenzylamides were omitted from the standards since they were generally obscured by ultraviolet-absorbing material that was extracted from the stained polyacrylamide gel. However, independent standards indicate the first two peaks of radioactivity correspond to these components. The experimental precision is limited by the 80–90% efficiency of the derivatization and extraction steps (estimated by the radioactivity found in the polyacrylamide gel and aqueous phase after the extractions) and by the assumption that the same fraction of the total enzyme is found in peptide I and peptide II. An equilibrium constant for transesterification can be calculated as the ratio of the radioactivity obtained from the two peptides, which should be equal to the ratio of intermediates on the active cysteine and 4′-phosphopantetheine. These ratios are given in columns 4 and 8 of Table III: for carbon chain lengths shorter than C_{12}, the equilibrium constant is greater than 1; it becomes progressively smaller for C_{14}, C_{16}, and for C_{18}, where the equilibrium constant is ~0.05.

The relative abundances of the saturated intermediates detected on the trypsinized enzyme with increasing incubation times are given in Table IV. All of the intermediates, from C_4 to C_{18}, were detected at both times, and the concentrations of the intermediates from C_6 to C_{14} did not vary by more than 2-fold. As the incubation time increased, the relative concentration of the C_4 intermediate decreased, whereas the relative concentrations of the C_{16} and C_{18} intermediates increased. These results are similar to those presented in columns 5 and 9 of Table III. The results obtained with native enzyme are similar, except very little C_{16} intermediate was observed, and no C_{18} intermediate was detected.

Relative Abundance of Intermediates with Limiting NADPH. The different stereospecificity at C-4 of NADPH for the two reduction steps in the fatty acid synthase cycle can be used to determine the relative abundance of the unreduced (3-keto) and once reduced (3-hydroxy and 2,3 unsaturated) intermediates. The radioactivity of the intermediates formed by reduction of the acetoacetyl-enzyme with various concentrations of limiting NADPH, followed by reduction with (*S*)- or (*R*)-[4-^{3}H]NADPH (Figure 2), is presented in Table V. The ratios of radioactivity with (*S*)- and (*R*)-[4-^{3}H]NADPH, corrected with the control experiments (Figure 2), are given in columns 4, 7, 10, and 13. The control experiments correct for the difference in specific radioactivity of the (*S*)- and (*R*)-[4-^{3}H]NADPH. The results in columns 11–13 utilized malonyl as the primer, rather than acetoacetyl, and indicate the method of priming does not influence the ratio. These results indicate that for all chain lengths, the amount of 3-keto intermediate is considerably greater than the amounts of 3-hydroxy and enoyl intermediates. The precision of these results is limited because separate samples were necessarily used for each experiment. Nevertheless, the 3-keto intermediates represent 70–100% of the total.

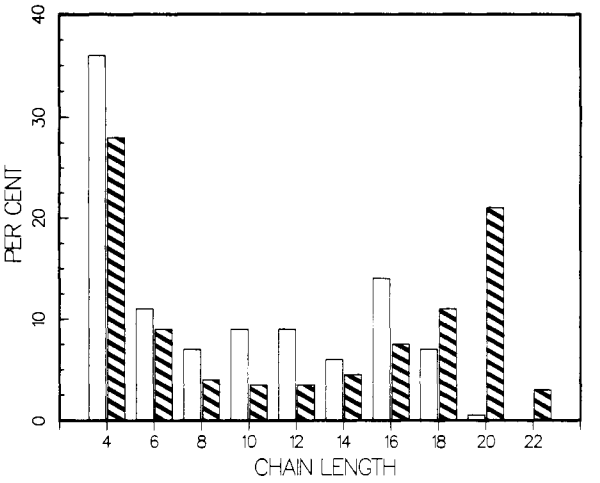

FIGURE 3: Relative concentrations of the saturated intermediates (open bars) and 3-keto intermediates (hatched bars) isolated when acetoacetyl-enzyme incorporates 2.7 malonyl (limiting malonyl-CoA) or 7.0 NADPH (limiting NADPH) equiv, respectively. The experiments were carried out with acetoacetyl-trypsinized fatty acid synthase in 0.1 M sodium phosphate, pH 7.0, and 1 mM EDTA.

The relative abundance of the different chain length 3-keto intermediates determined from three experiments with an average incorporation of 7 equiv of NADPH is shown in Figure 3 together with the distribution obtained with limiting malonyl-CoA. (The equivalents of NADPH initially present are calculated by summing the number needed to produce the

Table V: Relative Abundance of Intermediates[a]

carbon chain length	intermediates (cpm) with 1.1 NADPH equiv total	keto	% keto[b]	intermediates (cpm) with 2.2 NADPH equiv total	keto	% keto[b]	intermediates (cpm) with 6.5 NADPH equiv total	keto	% keto[b]	intermediates (cpm) with 2.0 NADPH equiv[c] total	keto	% keto[b]
4	6550	12010	86	3180	5160	82	4990	11000	100	1522	3450	110
6	2450	3540	68	2780	4310	79	1300	2990	110	840	1400	78
8	690	1210	82	1350	2170	82	860	1500	82	390	800	96
10	190	380	93	430	840	99	620	1370	100	100	250	120
12	86	190	100	220	410	95	790	1470	87	86	150	82
14	49	110	100	85	160	95	1100	2110	90	170	250	69
16	30	63	*d*	50	72	*d*	1400	2430	81	260	380	68
18	15	48	*d*	29	59	*d*	1820	3660	94	280	450	75
20	50	58	*d*	<20	<20	*d*	2060	3470	79	960	1530	74
22	<20	<20	*d*	<20	<20	*d*	260	510	92	640	1210	88
24	<20	<20	*d*	<20	<20	*d*	<20	<20	*d*	80	130	76

[a] Approximately 1 μM trypsinized acetoacetyl-enzyme, 50 μM malonyl-CoA, 0.1 M sodium phosphate, pH 7.0, 1 mM EDTA, and varying NADPH concentrations. [b] Corrected for ratio of specific activities of (*R*)- and (*S*)-[4-^{3}H]NADPH: 2.14 for data sets 1, 3, and 4; 1.97 for data set 2. [c] With malonyl-CoA as primer rather than acetoacetyl-labeled enzyme. [d] Amount of radioactivity is too small to determine a meaningful ratio.

amount of observed products.) Unreacted acetoacetyl-enzyme is the predominant species, representing an average of 28% of the total intermediates. The relative concentrations of the C_6–C_{14} species decrease progressively to ~4% for the C_{12} and C_{14} intermediates. The concentration of the long-chain intermediates increases until C_{20}, which represents an average of 21% of the intermediates. When only 1.1–2.2 equiv of NADPH was consumed, the major intermediate was unreacted acetoacetyl-enzyme, while the concentration of the longer chain intermediates progressively decreased through C_{20}, which was barely detectable.

The distribution of intermediates of a control experiment in which the acetoacetylation of the enzyme was omitted (columns 11–13 of Table V) is very different from the experiment with acetoacetyl-enzyme (columns 5–7 of Table V), although the ratio of NADPH to total enzyme was identical. When the enzyme is treated with 0.4 M hydroxylamine, pH 7.0, for 1 h at room temperature after being denatured in guanidine hydrochloride, >95% of the ratioactivity is removed. This indicates the saturated intermediates are essentially all thio esters.

Conversion of [1-^{14}C]Acetyl- and [2-^{14}C]Malonyl-Enzyme to Product. By addition of [1-^{14}C]acetyl- or [2-^{14}C]-malonyl-enzyme to a solution containing a large excess of nonradioactive acetyl-CoA and malonyl-CoA and a slight excess of NADPH, the fraction of the radioactive label converted to long-chain fatty acids can be determined. Under the standard reaction conditions, 30% of the [1-^{14}C]acetyl label and 6.7% of the [2-^{14}C]malonyl label are converted to product (averages of three experiments). Since only 1–2% of the nonradioactive malonyl-CoA in the reaction solution is converted to product, the radioactivity detected in the product cannot be attributed to malonyl label that is transferred from the enzyme to free CoA, with the resulting [2-^{14}C]malonyl-CoA being converted to product. Two control experiments indicated that only 0.9% of [2-^{14}C]malonyl-CoA added directly to the reaction solution was converted to product and that only 2% of the [2-^{14}C]malonyl was converted to product in the absence of acetyl-CoA. Two pools of cyclohexane extracts were collected to demonstrate the efficiency of the extraction. The first cyclohexane extract pool typically had over 90% of the total counts extracted by cyclohexane, indicating that the radioactive product is more soluble in cyclohexane than in water. This result is expected for long-chain fatty acids, but not for acetate or malonate.

Discussion

Isotope-trapping experiments (Krishnaswamy et al., 1962) provide a sensitive tool for determining the mechanism of reactions. McCarthy et al. (1983), on the basis of substrate labeling experiments, proposed that both the acetyl and malonyl moieties were transferred from their CoA thio esters to a unique serine prior to being transferred to the 4′-phosphopantetheine thiol, with the acetyl moiety being subsequently transferred to the active cysteine. This implies a mechanism in which the addition of substrates is ordered, with the acetyl moiety adding first. Soulié et al. (1983), on the basis of kinetic studies, also proposed an ordered addition of substrates. However, since significant quantities of both [1-^{14}C]acetyl- and [2-^{14}C]malonyl-labeled enzyme are converted to product, a random component to the initiation reaction must exist in addition to the ordered mechanism. Several alternative interpretations of the data have been ruled out by control experiments. Incorporation of the radioactive label into product cannot be due to exchange with medium malonyl-CoA since direct addition of radioactively labeled malonyl-CoA to the quenching solution resulted in <1% incorporation of radioactivity into the product. Initiation of the reaction due to contamination of [2-^{14}C]malonyl-CoA by decarboxylation or by endogenous acetyl-CoA is unlikely since omission of acetyl-CoA from the trapping solution decreased the amount of [2-^{14}C]malonyl-enzyme converted to product by more than 70%. On the other hand, the incorporation of radioactivity into product is probably as small as it is because both acetyl and malonyl moieties can covalently bind to sites that are not catalytically important (Nixon et al., 1970), binding to the serine hydroxyl is labile (Cardon & Hammes, 1983), and free CoA rapidly removes covalently bound acetyl and malonyl groups (Stern et al., 1982; Cognet & Hammes, 1983).

Relatively little information is available concerning the location of reaction intermediates on fatty acid synthase, although the location of acetyl groups on the 4′-phosphopantetheine and the active cysteine, and malonyl and 3-ketooctanoyl on the former, is well established (Kumer & Srinivasan, 1981). Also, long-chain fatty acids have been found bound almost exclusively to the 4′-phosphopantetheine (Libertini & Smith, 1979). The results reported here indicate that the 3-hydroxybutyryl intermediate is bound almost solely to the 4′-phosphopantetheine and that the saturated intermediates from C_4 to C_{14} may be found on both the active cysteine and 4′-phosphopantetheine. This evidence is consistent with the assumption that the two reduction reactions and the dehydration reaction occur when the intermediates are bound to the 4′-phosphopantetheine. A shortcoming of these experiments is that benzylamine does not derivatize the unsaturated intermediate: for example, benzylamine does not convert [3-^{3}H]crotonyl-CoA to [3-^{3}H]crotonylbenzylamide (unpublished results). In a similar vein, *N*-acetyl-*S*-crotonylcysteamine is resistant to hydrolysis by strong base (Sedgwick & Morris, 1980). However, this problem cannot dramatically influence the conclusions reached since over 80% of the radioactivity is extracted from the polyacrylamide gels by derivatization with benzylamine. This is consistent with the fact that for the hydration of carbon–carbon double bonds, the hydrated form is typically favored by a factor of 3–4 [cf. Frieden et al. (1957) and Stern (1955)].

When acetoacetyl-labeled fatty acid synthase is incubated with limiting amounts of either malonyl-CoA or NADPH and an excess of the other, the elongation reaction proceeds until the limiting substrate is exhausted. The resulting distribution of intermediates can be used to infer the chain length dependence of the second-order rate constants for the condensation and reduction reactions. Because the enzyme is primed with acetoacetyl-CoA, the initiation reactions are bypassed, and because the enzyme is trypsinized, the thioesterase is eliminated. For limiting malonyl-CoA, the reaction sequence can be represented as

$$\begin{aligned} E\text{-}C_4 + \text{malonyl-CoA} &\xrightarrow{k_1} E\text{-}C_6 \\ E\text{-}C_6 + \text{malonyl-CoA} &\xrightarrow{k_2} E\text{-}C_8 \\ &\vdots \\ E\text{-}C_{20} + \text{malonyl-CoA} &\xrightarrow{k_8} E\text{-}C_{22} \end{aligned} \qquad (2)$$

For limiting NADPH, the reaction sequence can be written as

$$\begin{aligned} E\text{-}C_4' + 2\text{NADPH} &\xrightarrow{k_1'} E\text{-}C_4'' + \text{NADPH} \xrightarrow{k_1''} E\text{-}C_6' \\ E\text{-}C_6' + 2\text{NADPH} &\xrightarrow{k_2'} E\text{-}C_6'' + \text{NADPH} \xrightarrow{k_2''} E\text{-}C_8' \\ &\vdots \\ E\text{-}C_{20}' + 2\text{NADPH} &\xrightarrow{k_8'} E\text{-}C_{20}'' + \text{NADPH} \xrightarrow{k_8''} E\text{-}C_{22}' \end{aligned} \qquad (3)$$

In these equations, the saturated intermediates are unprimed, the 3-keto intermediates have a single prime, and the 3-

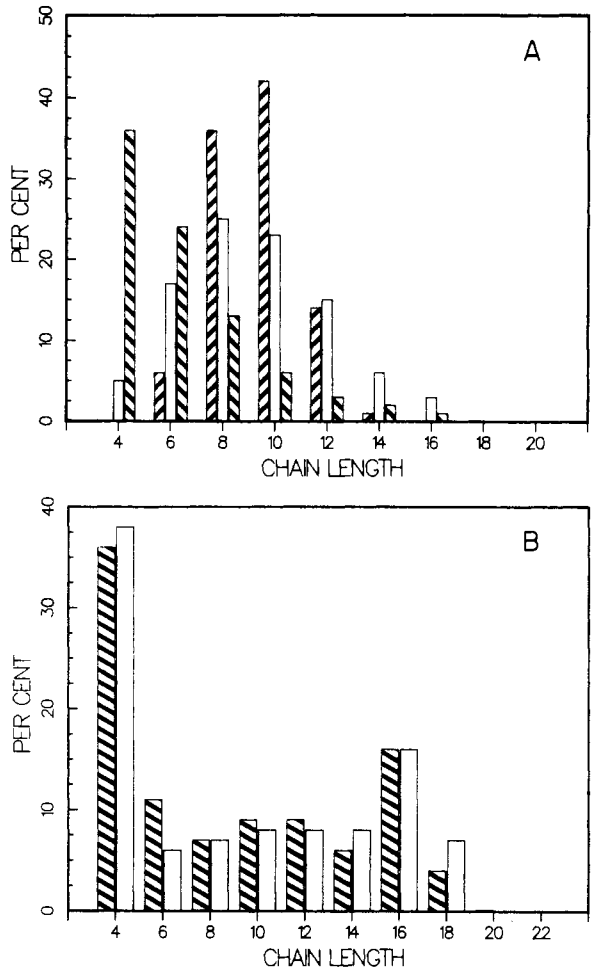

FIGURE 4: Relative concentrations of saturated intermediates formed with limiting malonyl-CoA. (A) Calculated distributions according to eq 2 obtained with 2.7 malonyl equiv and with $k_n = 0.5k_{n-1}$ (▨), $k_n = k_{n-1}$ (□), and $k_n = 2k_{n-1}$ (▩). (B) Comparison of experimental distribution (▩) with calculated distribution (□). For the calculations, $7k_1 = k_2 = k_3 = k_4 = k_5$ and $k_{6-8} = 7k_1K/(1 + K)$ where K is the experimentally determined transesterification equilibrium constant. The experiments were carried out with acetoacetyl-trypsinized fatty acid synthase in 0.1 M sodium phosphate, pH 7.0, and 1 mM EDTA.

hydroxy intermediates have a double prime. The second-order reactions are assumed to be irreversible because of the large favorable equilibrium constants and because they are coupled to reactions with a large excess of the other substrate. The simplification also is made that intramolecular interconversions can be included in the second-order rate constant. This is true if the intramolecular reactions are rapid relative to the second-order process. This occurs at sufficiently low concentrations of the limiting substrate, if other species are present in relatively low concentrations or if the other species are present in a steady state. The rate equations for both mechanisms can be numerically integrated and the distribution of chain lengths in the intermediates determined for specific relative values of the second-order rate constants.

Figure 4A presents the calculated distribution of chain lengths with an initial malonyl-CoA/E–C_4 ratio fo 2.7. If all of the condensation rates constants are assumed to be equal, a Poisson distribution would result, as shown by the middle bar in each set. If each successive condensation rate constant is smaller by a factor of 0.5, the standard deviation around the mean is decreased, as shown by the first bar in each set. If each successive condensation rate constant is larger by a factor of 2, the distribution decreases exponentially, as illustrated by the third bar of each set.

The experimental distribution of chain lengths for the trypsinized enzyme obtained from the data in Table IV at 120 s is shown in Figure 4B as the first bar in each set. The approximately equal amounts of C_6–C_{14} intermediates suggest that k_2–k_6 are roughly equal and larger than k_1. (Under steady-state conditions, the concentrations of the intermediates are inversely proportional to the condensation rate constants for their formation. The similar distributions at 15 and 120 s in Table IV suggest this condition has been achieved. This is consistent with the condition that $k_2 - k_5 > k_1$.) The second bar in each set in Figure 4B was generated by assuming $k_2 - k_5 = 7k_1$. The rate constants k_6–k_8 were multiplied by the fraction of intermediates on the cysteine since this is presumably the reactive species. Specifically, $k_n = k_5K_n/(1 + K_n)$ (n = 6, 7, and 8) where K_n is the transesterification equilibrium constant obtained from column 8 and Table III. This distribution is in reasonable agreement with the observed data. Note that the prominent buildup of C_{16} and C_{18} can be generated by assuming only that the measured change in the transesterification equilibrium constant reduces the fraction of saturated intermediate available to the condensation reaction; i.e., the intrinsic condensation rate constant need not change at long chain lengths.

The results with native enzyme presented in Table IV indicate that the C_6–C_{14} intermediates are present in nearly the same proportions as with the trypsinized enzyme. Since the concentrations of C_{12} and C_{14} intermediates observed after 15 and 120 s are similar, the thioesterase must have a very strong chain length specificity.

The results in Table V show that for each carbon chain length, the 3-keto intermediate represents over 70% of the total; this indicates that for all chain lengths the reduction of the unsaturated intermediates must be at least 2 times faster than the reduction of the 3-keto intermediate. These results extend the end product analysis of Sumper et al. (1969) and of Libertini & Smith (1979), who found that only 3-keto long-chain fatty acids were produced by the yeast and rat liver enzymes, respectively, when low NADPH concentrations were employed. These data are also consistent with the reduction rate constants for acetoacetyl-enzyme (Cognet et al., 1983) and crotonyl-enzyme (Cognet & Hammes, 1985).

The chain length distribution of the 3-keto intermediates observed with low NADPH concentrations is similar to the distribution of saturated intermediates observed with limiting malonyl-CoA, as shown in Figure 3. The same conclusions may be drawn: the initial reduction reaction is slower than the subsequent reactions, and the concentrations of the intermediates, C_4–C_{14}, are inversely proportional to their relative rate constants. The major difference between the distributions of the 3-keto and saturated intermediates is that the 3-keto distribution reaches a peak at C_{18}–C_{20}, rather than at C_{16}. However, this implied decrease in the rate of the reduction reaction at long chain lengths may be overestimated. The large decrease in the transesterification equilibrium constant suggests that the unimolecular rate of acyl transfer from the 4′-phosphopantetheine to the cysteine may decrease significantly and could become slower than many of the second-order rates. Thus, the analysis presented may be oversimplified. The relative rates of interconversion of intermediates observed in this study are well suited to produce palmitate specifically and efficiently. The increasing rates at medium chain lengths minimize the concentration of those intermediates that are subject to hydrolysis by the thioesterase. Similarly, maintaining the rapid reduction rates through the C_{16} intermediate reduces the probability of producing 3-keto fatty acids. The

transesterification equilibrium constant and the thioesterase specificity apparently contribute more to the product specificity than the condensation rates. The turnover number for the thioesterase is not known. However, since the turnover numbers of the overall reaction and the other enzymes can be estimated from this and previous (Cox & Hammes, 1983; Cognet & Hammes, 1985) work, a rough estimate for this turnover number of 10–100 s^{-1} can be made. Apparently, all of the individual turnover numbers are similar in magnitude so that a single rate-determining step does not exist for the overall reaction.

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Isolation and Characterization of a New Endo-β-galactosidase from *Diplococcus pneumoniae*†

Michiko N. Fukuda

Cancer Research Center, La Jolla Cancer Research Foundation, La Jolla, California 92037

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ABSTRACT: An endo-β-galactosidase, which hydrolyzes the internal β-galactosidic linkages of R→GlcNAc(or GalNAc)β1→3Galβ1→4GlcNAc(or Glc), was isolated from the culture supernatant of *Diplococcus pneumoniae*. The enzyme, named endo-β-galactosidase D_{II}, hydrolyzed linear *N*-acetyllactosamine repeating structures in glycolipids and glycopeptides to release oligosaccharides. The specificity of endo-β-galactosidase D_{II} is the same as that of *Escherichia freundii* endo-β-galactosidase as far as described above, but the following differences between these two enzymes were found: (1) Branched lactosaminyl glycolipids and H-antigenic glycolipids were resistant to endo-β-galactosidase D_{II}, even when linear structure was present at the inner part. (2) Throughout the enzymic hydrolysis, endo-β-galactosidase D_{II} released mostly small oligosaccharides (tetra-, tri-, and disaccharides) from substrates, suggesting that the enzyme split off the oligosaccharides stepwise from the nonreducing terminal. (3) Lactosaminoglycans were partially hydrolyzed by endo-β-galactosidase D_{II} to produce small oligosaccharides as the major product and residual glycopeptides. The residual glycopeptides were readily hydrolyzed by *E. freundii* endo-β-galactosidase to produce various sizes of oligosaccharides. (4) Keratan sulfate was not degraded by endo-β-galactosidase D_{II}. These properties of endo-β-galactosidase D_{II} characterize it as a new endo-β-galactosidase with a unique specificity.

Lactosaminoglycan glycoproteins and lacto-series glycolipids are characterized by repeating the *N*-acetyllactosamine sequence $(GlcNAc\beta1\rightarrow3Gal\beta1\rightarrow4)_n$ and by their susceptibility to endo-β-galactosidase (Fukuda et al., 1978b, 1979b). These glycoconjugates are responsible for cell surface antigens, such as Ii and ABO blood group systems. The previous studies have shown that expression of these antigens is regulated under

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