PURIFICATION AND CHARACTERIZATION OF A NATURALLY OCCURRING ACTIVATOR OF CHOLESTEROL BIOSYNTHESIS FROM $\Delta^{5,7}$-CHOLESTADIENOL AND OTHER PRECURSORS[1,2]

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The reduction of $\Delta^{5,7}$-cholestadienol to cholesterol by the microsomal fraction of rat liver homogenates is specifically stimulated 4-fold or greater, in the presence of excess NADPH, by an activator isolated from the high speed supernatant fraction. This activator was purified by heat treatment, $(NH_4)_2SO_4$ fractionation, and gel filtration. It is inactivated by trypsin hydrolysis. The results demonstrate that the activator is a heat stable protein capable of binding $\Delta^{5,7}$-cholestadienol, which in bound form is readily converted to cholesterol by microsomal $\Delta^{5,7}$-sterol Δ^{7}-reductase. Similar findings were obtained with other precursors of cholesterol and suggest a unique biosynthetic function for the protein as a vehicle for water insoluble precursors of cholesterol.

It is now well established that $\Delta^{5,7}$-sterol Δ^{7}-reductase of rat liver requires NADPH as cofactor (Figure 1) (1-4). Previous work from this laboratory also showed that formation of cholesterol from $\Delta^{5,7}$-cholestadienol occurs in the presence of the lower half of the 105,000 x g supernatant fraction of liver homogenates. Furthermore, combination of the upper half of the 105,000 x g superna-

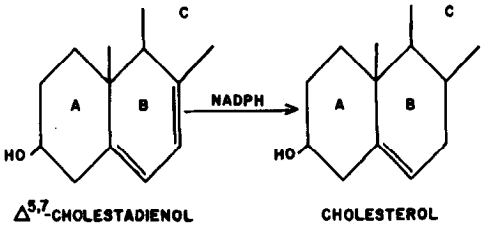

Figure 1. Conversion of $\Delta^{5,7}$-cholestadienol to cholesterol by $\Delta^{5,7}$-sterol Δ^{7}-reductase.

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2. Systematic names of the sterols referred to in the text by their abbreviated names are: $\Delta^{5,7}$-cholestadienol, $\Delta^{5,7}$-cholestadien-3β-ol; Δ^{7}-cholestenol, Δ^{7}-cholesten-3β-ol; cholesterol, Δ^{5}-cholesten-3β-ol.

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tant (exhibits none or barely measurable reductase activity) and washed microsomal (exhibits low reductase activity) fractions produces levels of activity comparable to those observed in the lower half of the supernatant fraction (a mixture of solubilized or slowly sedimenting microsomes and the supernatant fraction) (2-5). The purpose of this report is to describe certain quanitative aspects of activation of microsomal Δ^7-reductase by the upper supernatant fraction; the purification and characterization of the activating component; and its probable mechanism of action.

METHODS

Preparation from rat liver homogenates of Fraction A (upper half of the 105,000 x g supernatant fraction); Fraction C (washed microsomal fraction); Fraction D (Fraction C, purified by solubilization, salt fractionation, and gel filtration) and enzymic assays (for cholesterol-4-^{14}C synthesis from synthetic $\Delta^{5,7}$-cholestadienol-4-^{14}C by passage through the dibromide derivative and for $\Delta^{5,7}$-cholestadienol-2,4-^{3}H synthesis from synthetic Δ^7-cholestenol-2,4-^{3}H by formation of the epiperoxide derivative) were as described in detail previously (4). The activator of microsomal Δ^7-reductase was purified from Fraction A by first heating an aliquot of Fraction A in 5% (w/v) $(NH_4)_2SO_4$ for 5 minutes at 100° under nitrogen. After removal of precipitated protein by filtration or centrifugation (5,000 x g, 30 minutes), the soluble protein was isolated either by precipitation with $(NH_4)_2SO_4$ (100% w/v) and passage through a column of Sephadex G-25 ((4) and Tables I-II) or by the procedure outlined in the conditions with Table III, yielding the highest level of purification. The purified activator is designated A_1 (Tables I-IV).

RESULTS AND DISCUSSION

The data of Figures 2 and 3 show that the high-speed supernatant fraction (A) is required in addition to NADPH for maximum conversion of $\Delta^{5,7}$-cholestadienol to cholesterol by purified microsomal Δ^7-reductase. Activation of Δ^7-reductase by Fraction A is usually 4-fold or greater. The optimum ratio of A protein to

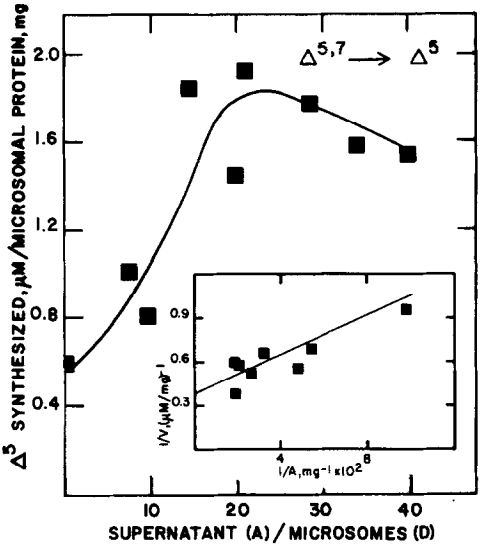

Figure 2. Activation of Microsomal Δ^7-Reductase by Varying Levels of the High-Speed Supernatant Fraction.

Reaction mixtures contained 0.1 M phosphate buffer, pH 7.3, 9.3 μM $\Delta^{5,7}$-cholestadienol-4-^{14}C, 1 mM NADPH, 1.2 mg purified microsomal Δ^7-reductase protein (Fraction D, "Methods" and (4)), and when present, high-speed supernatant protein (Fraction A, "Methods" and (4)) at the various levels indicated, in 2.8 ml final volume. After incubation for 45 minutes at 37° under nitrogen, reactions were stopped by addition of 2.8 ml 95% (v/v) ethanol. Assay for cholesterol-4-^{14}C (Δ^5) synthesis was by passage through the dibromide derivative (4).

purified microsomal (D) protein is approximately 21 (cf. Figs. 2 and 3). Under the conditions of the experiments (e.g. Fig. 2), the level of A protein required for half maximum Δ^7-reductase activity was 17 mg; the calculated maximum velocity was 2.8 μM cholesterol synthesized/mg microsomal protein (insert, Fig. 2). These results further demonstrate that Fraction A, in the absence of the microsomal preparation, exhibits little if any Δ^7-reductase activity (Fig. 3) while low reductase activity is seen with microsomes in the absence of A (Fig. 2). There is also a marked lag in the activation of Δ^7-reductase by increasing levels of A protein (Fig. 2), indicating possible subunit interactions during the activation process (cf. Table IV).

Related experiments determined that activation of microsomal Δ^7-reductase is specific for Fraction A, i.e. rat and human serum proteins, bovine plasma

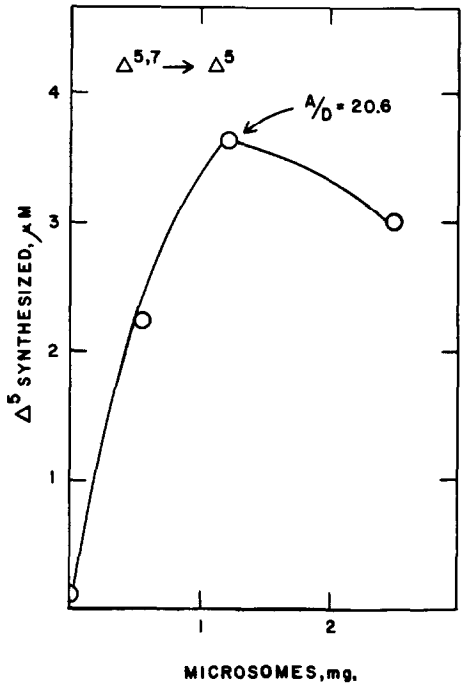

Figure 3. Activation of Varying Levels of Microsomal Δ^7-Reductase by the High-Speed Supernatant Fraction.

Reaction mixtures contained 0.1 M phosphate buffer, pH 7.3, 12.4 μM $\Delta^{5,7}$-cholestadienol-4-^{14}C, 1 mM NADPH, 25.8 mg high-speed supernatant protein (Fraction A), and, when present, purified microsomal Δ^7-reductase protein (Fraction D), at the various levels indicated, in 2.1 ml final volume. Incubations were for 30 minutes under nitrogen. Other conditions and assay for cholesterol-4-^{14}C (Δ^5) synthesis were as described with Figure 2.

albumin, and soybean trypsin inhibitor (Table II) will not substitute for Fraction A. Activator activity is present in the phosphate buffer (0.1 M) extract of rat liver acetone powder and has the same characteristics as Fraction A (6).

An important property permitting marked purification of the activator of microsomal Δ^7-reductase is its heat stability. Fraction A may be heated at 100° for one minute (Experiment 1, Table I) or for 10 minutes after purification (Experiment 2, Table I) without appreciable loss of activation ability. Strong evidence that the activator is a heat stable protein is presented in Table II; trypsin catalyzed proteolysis of the purified activator (A_1) resulted in nearly complete loss of activation ability. Additional evidence is that the

Table I

HEAT STABILITY OF THE ACTIVATOR OF MICROSOMAL Δ^7-REDUCTASE

Experiment	Activator Preparation(a)	Protein Ratio, Activator/Microsomes	Activation of Δ^7-Reductase(b)
			-fold
1	A	4.7	4.3
	A, 100°, 1 min.	4.0	4.0
2	A_1	0.3	3.0
	A_1, 100°, 1 min.	0.1	3.0

(a) Aliquots of the high-speed supernatant (Fraction A) or the purified activator (A_1) ("Methods" and (4)) were heated at 100° under nitrogen for the time indicated, followed by cooling to 4°, and pH adjustment to 7.4. In Experiment 1, Fraction A was heated for 1 minute and the coagulated protein resuspended by homogenization. In Experiment 2, purified activator (A_1) was heated for 10 minutes and the precipitate discarded.
(b) For activation assays, each reaction mixture contained 0.1 M phosphate buffer, pH 7.3, 11.3 μM $\Delta^{5,7}$-cholestadienol-4-^{14}C, 1 mM NADPH, 5.7 mg (Experiment 1) or 6.1 mg (Experiment 2) microsomal Δ^7-reductase protein (Fraction C, "Methods" and (4)), and the activator preparations indicated in 2.4 ml final volume. Different activator and microsomal preparations were used in the two experiments. Other conditions and assay for cholesterol-4-^{14}C synthesis were as given with Figure 2.

activator is nondialyzable and precipitated by perchloric acid. Furthermore, activation activity accompanies the protein during heat treatment, $(NH_4)_2SO_4$ fractionation, and gel filtration (Table III). The latter procedures result in a 300-fold purification of the heat stable protein (A_1), having a striking ability to activate Δ^7-reductase (e.g. 37-fold under the assay conditions with Table III). The level of activation observed is dependent on the ratio of A_1 protein to microsomal protein (cf. Figs. 2 and 3, for the unpurified activator) and substrate concentration. Kinetic studies further demonstrated that both the apparent K_m for $\Delta^{5,7}$-cholestadienol and maximum velocity of the Δ^7-reductase reaction increase with increasing ratios of A_1 to microsomal protein. It was also noted that sulfhydryl compounds (e.g. reduced glutathione, cysteine, and dithiothreitol) maintain the activation ability of A_1 during storage at

Table II

TRYPSIN HYDROLYSIS OF THE ACTIVATOR OF MICROSOMAL Δ^7-REDUCTASE

Activator Preparation[a]	Activation of Δ^7-Reductase[b]
	-fold
A_1	5.9
Trypsin treated A_1 plus Trypsin Inhibitor	1.2
Trypsin Inhibitor	1.5

(a) Purified activator (A_1) protein (2.2 mg), pH 8.9, was incubated at 37° with 0.24 mg trypsin (bovine pancreas, Sigma Type III, twice crystallized). Proteolysis was stopped by addition of 0.24 mg soybean trypsin inhibitor (Sigma Type I-S, twice crystallized), cooling to 4°, and pH adjustment to 7.4. A 40.5% decrease in A_1 protein resulted.
(b) For activation assays, each reaction mixture contained 0.1 M phosphate buffer, pH 7.3, 16.3 μM $\Delta^{5,7}$-cholestadienol-4-^{14}C, 1 mM NADPH, 6.8 mg microsomal Δ^7-reductase protein (Fraction C "Methods" and (4)), and when present, 1.7 mg A_1 protein; or trypsin treated A_1 protein plus trypsin inhibitor, just described; or 0.2 mg soybean trypsin inhibitor in 2.4 ml final volume. Other conditions and assay for cholesterol-4-^{14}C synthesis were as given with Figure 2.

Table III

PURIFICATION OF THE ACTIVATOR OF MICROSOMAL Δ^7-REDUCTASE

Activator Preparation	Activation of Δ^7-Reductase[a]	Total Activator Protein	Specific Activity[b]	Total Activity	Purification	Yield
	-fold	mg	μM/mg		-fold	%
A	5.2	2347.2	0.03	70.4	1.0	100
A_1[c] (M.W. ~ 16,000)	36.8	2.9	9.01	26.1	300	37

(a) Protein ratio, activator/microsomes, in assays was 3.6 for Fraction A and 0.3 for Fraction A_1. Other conditions were as described with Tables I-II, except that 5 mM reduced glutathione was also present in assay mixtures.
(b) Cholesterol-4-^{14}C synthesized, μM/mg A or A_1 protein; corrected for the low microsomal Δ^7-reductase activity in the absence of A or A_1.
(c) Activator protein (A_1) was prepared from the high-speed supernatant (Fraction A) as described in "Methods" and (4), except that only the heat stable protein precipitating between 40 and 80% (w/v) saturation with $(NH_4)_2SO_4$ was collected and passed through a column (1.5 x 90 cm) of Sephadex G-75 connected directly to a similar column. Elution was with 0.1 M phosphate buffer, pH 7.3 (18 ml/hour; 4.5 ml fractions) at 4°. Activator (A_1) activity was associated with the protein (molecular weight approximately 16,000) in column fractions 42-43. A low level of A_1 activity accompanied the protein in fractions 21-23 (molecular weight > 60,000) (cf. Table IV). Other fractions contained no detectable A_1 activity.

Table IV

SUBSTRATE BINDING BY PROTEIN ACTIVATOR OF CHOLESTEROL BIOSYNTHESIS

Activator Incubated with Substrate	Sterol Substrate	Protein Peak I[a] (M.W. >60,000)		Protein Peak II[a] (M.W. ~16,000)	
		Enzymic Activation[b,c]	Bound Sterol[d]	Enzymic Activation[b]	Bound Sterol[d]
		-fold	%	-fold	%
A, 1 min., 100°[e]	$\Delta^{5,7}$	9.6	78	1.9	2
	Δ^{7}	5.0	90	4.3	< 1
Protein Peak I[a]					
From A[f]	$\Delta^{5,7}$	9.5	78	--[h]	--[h]
From A_1[g]	$\Delta^{5,7}$	5.1	34	--[h]	--[h]
Protein Peak II[a]					
From A[f]	$\Delta^{5,7}$	--[h]	--[h]	1.6	3
From A_1[g]	$\Delta^{5,7}$	5.9	53	5.9	3

(a) From Sephadex G-75 column fractionation as described in the conditions with Table III.
(b) $\Delta^{5,7}$-Sterol Δ^{7}-reductase, with $\Delta^{5,7}$-cholestadienol-4-^{14}C as substrate; Δ^{7}-sterol Δ^{5}-dehydrogenase, with Δ^{7}-cholestenol-2,4-^{3}H as substrate. Activation assays were performed as described with Tables I-III and (4). Activation levels reflect variations in activator to microsomal protein ratios and substrate levels (cf. (c)) in the assays.
(c) Total substrate concentration in activation assays included bound sterol and was 20-30 μM for $\Delta^{5,7}$-cholestadienol-4-^{14}C experiments and 13 μM for Δ^{7}-cholestenol-2,4-^{3}H experiment.
(d) Expressed as percent of radioactivity applied to the Sephadex G-75 column with the protein to be fractionated.
(e) An aliquot of the high-speed supernatant (Fraction A) was heated for 1 minute at 100° as described in the conditions with Table I and coagulated protein removed by centrifugation. The supernatant was then incubated with $\Delta^{5,7}$-cholestadienol-4-^{14}C (1.1 mμmoles/mg protein) or Δ^{7}-cholestenol-2,4-^{3}H (0.4 mμmole /mg protein) for 15 minutes at 37°, followed by $(NH_4)_2SO_4$ precipitation (40-80% saturation) and gel filtration according to the conditions with Table III. The column effluent was assayed for protein bound radioactivity and enzymic activation (4).
(f) Peaks I and II obtained by gel filtration on Sephadex G-75 (Table III) of untreated Fraction A were each incubated with $\Delta^{5,7}$-cholestadienol-4-^{14}C (2.2 mμmoles/mg Peak I protein or 41 mμmoles/mg Peak II protein) for 30 minutes at 37° and refractionated on Sephadex G-75 (Table III). The column effluent was assayed for protein bound radioactivity and activation of Δ^{7}-reductase.
(g) Peaks I and II obtained by preparation of purified activator (A_1) as described in the conditions with Table III were incubated with $\Delta^{5,7}$-cholestadienol-4-^{14}C (127 mμmoles/mg Peak I protein or 128 mμmoles/mg Peak II protein), refractionated and assayed as above[f].
(h) No protein or radioactivity detected.

4° or purification steps (Table III). These agents do not activate Δ^7-reductase in the absence of A_1.

The requirement of A_1 protein for full microsomal Δ^7-reductase activity indicates that Δ^7-reductase should be classified as a member of the growing list of enzymes requiring more than one protein for their maximum activity (7). Most of these are bacterial enzymes and only one mammalian enzyme is well characterized (8). The probable role of A_1 in cholesterol biosynthesis is demonstrated by the data of Table IV. Sterol substrates ($\Delta^{5,7}$-cholestadienol or Δ^7-cholestenol) remain bound to the heat stable activator protein throughout its purification (as described with Table III). Bound substrates are associated with the high molecular weight ($>60,000$) species of A_1 and in bound form are readily converted to products by microsomal enzymes. The low molecular weight ($\sim 16,000$) species of A_1 arises during purification of A_1 (i.e. in the absence of bound substrate, Table III). In the process of binding substrate, the low molecular weight species aggregates to the high molecular weight species, the form of the activator in the unpurified high-speed supernatant (Fraction A). Similar studies (i.e. Table IV) showed that squalene and other water insoluble precursors of cholesterol are bound by A_1 while 3β-hydroxy steroids, not cholesterol precursors, are not bound (9). In addition, the substrate binding is noncovalent, i.e. bound sterols are readily extracted from A_1 into organic solvents. We are presently determining optimum conditions for conversion of low molecular weight A_1 into its higher aggregate and the affinity constants of A_1 and sterol precursors of cholesterol. Nonactivator protein (e.g. bovine serum albumin) will bind sterols, but with lower affinity than A_1.

The findings reported here and previous work describing activation by Fraction A of numerous microsomal enzymic steps in cholesterol biosynthesis and inhibition of activation of these steps by drugs blocking cholesterol synthesis suggest a common role for A_1 protein as a vehicle for water insoluble precursors of cholesterol (3-5, 9-11) (Fig. 4). This proposal is further supported by observations that specific enzymic steps (e.g. Δ^8— Δ^7-isomerase; Δ^{24}-reductase, Δ^5-

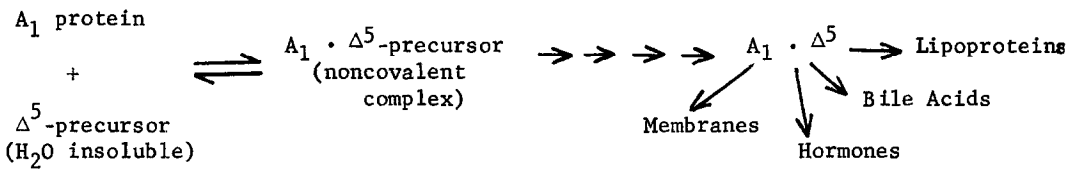

Figure 4. Role of Activator Protein (A_1) during Cholesterol (Δ^5) Synthesis by Microsomal Enzymes.

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dehydrogenase, Δ^7-reductase) are irreversible (3) and that it is not always possible to "trap" sterol intermediates during enzymic conversion to cholesterol (12).

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