

Regulation of Microsomal Stearoyl-Coenzyme A Desaturase

PURIFICATION OF A NON-SUBSTRATE-BINDING PROTEIN THAT STIMULATES ACTIVITY

By Dean P. JONES* and James L. GAYLOR†

*Department of Biochemistry, Emory University School of Medicine, Atlanta, GA 30322, U.S.A., and

†Department of Biochemistry, University of Missouri, Columbia, MO 65211, U.S.A.

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Crude cytosolic fraction from rat liver was examined for proteins that may be involved in regulation of microsomal stearoyl-CoA desaturase activity. Gel filtration revealed the presence of several components that either stimulate or inhibit this enzyme. In addition, other components bind the acyl-CoA substrate, thus affecting observed activities *in vitro*. A protein that stimulates stearoyl-CoA desaturase but does not bind substrate was purified approx. 1100-fold. The purified protein had no visible absorption spectrum and an approximate mol.wt. of 26 500. Maximal stimulation of desaturase activity occurred with less than 2 μ M purified protein. The protein was heat-labile and exhibited neither catalase nor glutathione peroxidase activity. Addition of the cytosolic protein produced no effect on the desaturase reaction stoichiometry; the proportions O₂ consumed/NADH oxidized/stearoyl-CoA desaturated remained 1:1:1. Because the K_m' for stearoyl-CoA was also unchanged by addition of the cytosolic protein, no change in substrate affinity was suggested. Furthermore addition of the cytosolic protein also produced no effect on desaturase inhibition by oleoyl-CoA, which suggested that the protein does not simply relieve apparent product inhibition. These results indicate that, in analogy to other cytosolic proteins that stimulate microsomal oxidase activities, the protein may have a regulatory function, perhaps related to activity modulation via organization of the multienzymic desaturase in the membrane.

Stearoyl-CoA desaturase is a microsomal oxidase system required for biosynthesis of oleic acid. Three protein components of this system (cytochrome *b*₅ reductase, cytochrome *b*₅ and the terminal oxidase) have been resolved, and an enzymically active desaturase has been reconstituted from the purified components (Strittmatter *et al.*, 1974; Enoch *et al.*, 1976). However, biochemical regulation of the metabolic activity of desaturase is not understood. Jeffcoat *et al.* (1976, 1977) found that desaturase activity could be stimulated by a basic cytoplasmic protein that binds substrate. This protein was recognized to be ligandin, which appears to stimulate activity by altering the apparent K_m for substrate. Other proteins that stimulate microsomal desaturase activity *in vitro* include bovine serum albumin (Jeffcoat *et al.*, 1976, 1977), catalase (Baker *et al.*, 1976), cytochrome *b*₅ (Jansson & Schenkman, 1975) and a protein extracted from isolated microsomal fractions (Catala *et al.*, 1975). Stimulation by proteins such as serum

albumin or catalase may be considered to be assay-specific, since this stimulation occurs only under certain requisite assay conditions, but is of questionable physiological significance *in vivo*. However, cytoplasmic substrate-binding proteins, such as ligandin or sterol carrier proteins of cholesterol biosynthesis (Scallen *et al.*, 1971; Ritter & Dempsey, 1971), may be constitutive components of enzyme systems, since they may promote catalytic efficiency by improving the apparent K_m .

Regulatory proteins that neither bind substrate nor affect K_m have not been identified for stearoyl-CoA desaturase. Grossly, desaturase shares several properties with another biosynthetic microsomal oxidase, methylsterol oxidase, which catalyses the oxidative removal of the 4 α -methyl group during the biosynthesis of cholesterol, and each differs significantly from the microsomal mixed-function oxidases that are cytochrome *P*-450-dependent. For example, both desaturase and demethylase are: more active with NADH than with NADPH (Oshino *et al.*, 1966; Gaylor & Mason, 1968); inhibited by either relatively low cyanide concentrations (Oshino *et al.*, 1966; Gaylor & Mason, 1968) or starvation (Oshino & Sato, 1972; Gaylor *et al.*, 1973); stimulated by either

Abbreviations used: desaturase is used for the multi-component enzyme system that catalyses the oxidative insertion of the double bond in position 9,10 of stearoyl-CoA. Similarly, demethylase is used for the multistep oxidation of the 4 α -methyl group of cholesterol precursors.

recovery from starvation (Oshino & Sato, 1972; Gaylor *et al.*, 1973) or the feeding of diets devoid of end products (Oshino & Sato, 1972; Holloway & Holloway, 1975); unaffected by CO (Gaylor & Mason, 1968; Oshino *et al.*, 1971; Wilson *et al.*, 1976); responsive to changes in hormonal balance (Gaylor *et al.*, 1973; Salmon & Hems, 1975). Gaylor & Delwiche (1976) purified a soluble protein that stimulates microsomal 4-methylsterol oxidase activity. End-product inhibition of this microsomal oxidase and also of hydroxymethylglutaryl-CoA reductase can be observed when the cytosolic protein is added to liver microsomal preparations. Because of the similarities of this system to desaturase, we undertook a fractionation of the post-microsomal supernatant fraction of rat liver homogenate in order to examine the nature of cytosolic regulatory components for desaturase. Isolation and characterization of a soluble protein that stimulates desaturase but neither affects methylsterol oxidase nor binds stearoyl-CoA are reported here.

Experimental

Materials

The following were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.): stearoyl-CoA (grade II), oleoyl-CoA, scopoletin (7-hydroxy-6-methoxycoumarin), β -NADH (grade III), β -NAD⁺ (grade III), α -NADH (grade II), sodium pyruvate (type II), lactate dehydrogenase (type II) and glucose oxidase (type V). Horseradish peroxidase was purchased from P-L Biochemicals (Milwaukee, WI, U.S.A.). Calcium phosphate gel (A-grade, lot 510005) and glutathione (A-grade, reduced) were purchased from Calbiochem (La Jolla, CA, U.S.A.). Catalase was from Worthington (Freehold, NJ, U.S.A.). Triton WR-1339 was from Ruger Chemical Co. (Irvington-on-Hudson, NJ, U.S.A.). [¹⁴C]Stearoyl-CoA (62 mCi/mmol), [^{9,10-³H}]stearoyl-CoA and [¹⁴C]oleoyl-CoA were purchased from New England Nuclear (Boston, MA, U.S.A.). Sephadex G-100 and DEAE-Sephadex were purchased from Pharmacia (Piscataway, NJ, U.S.A.). Phosphatidylcholine (Sigma) (10mg/ml of 0.1M-potassium phosphate buffer, pH7.4) was sonicated at 4°C for approx. 20min until the suspension clarified. Additions of 10 μ l/ml of incubation volume were made before protein additions. All other chemicals were at least reagent grade.

Tissue preparations

Control rats (male, Sprague-Dawley, 150–250g) were fed on rat chow *ad libitum*. Rats were decapitated (09:00–10:00h), and blood was removed from livers by perfusion *in situ* with 50ml of cold 0.25 M-sucrose. Livers were removed, minced in 3 vol. of 50mM-

potassium phosphate buffer (pH7.4 and containing 250mM-NaCl, 5mM-EDTA and 1mM-glutathione), and homogenized with a TenBroeck homogenizer (eight to ten strokes). Cell debris and more-dense organelles were removed from the suspension by centrifugation at 12000g for 20min. Microsomes (microsomal fraction) were sedimented from the resulting postmitochondrial supernatant fraction at 105000g for 1h. The resulting microsomal pellet was suspended in fresh buffer by homogenization, and washed microsomes were suspended in fresh 0.1M-potassium phosphate buffer, pH7.4, to a final concentration of 10–20mg of protein/ml. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a protein standard. Microsomes were used fresh except as indicated.

Radiochemical assay of stearoyl-CoA desaturation

Since several apparent means of stimulation of desaturase activity seemed possible and many of these related only to indirect assays, the laborious, but accurate, direct radioassay of [¹⁴C]oleoyl-CoA formation was used. The more rapid assay of ³H₂O from [^{9,10-³H}]stearoyl-CoA (Talamo & Bloch, 1969) was avoided because of possible activity changes due to kinetic isotope effects (Enoch *et al.*, 1976; Seifried & Gaylor, 1976), and assay based upon either oxidation/reduction changes (Oshino *et al.*, 1971; Strittmatter *et al.*, 1972) or O₂ consumption was avoided because of possible changes in alternative electron donors or acceptors. On the other hand, the ¹⁴C radioassay assumes equivalent extraction of stearic acid and oleic acid. If addition of supernatant proteins selectively alters the extraction of either the substrate or product, the assay is invalidated. To test this, two experiments were performed. First, [^{9,10-³H}]stearoyl-CoA and [¹⁴C]oleoyl-CoA were added to incubations of microsomes (1 mg/ml) with and without the crude cytosolic fraction (1 mg/ml); the ratios of recovered radiolabelled acids were the same for zero-time incubations. Second, 5min incubations of microsomes (1 mg/ml) with and without cytosolic fraction (1 mg/ml) containing [¹⁴C]stearoyl-CoA were carried out. Immediately before termination of the incubation by addition of methanolic KOH, [^{9,10-³H}]stearoyl-CoA was added. The resultant ratio of [³H]stearic acid to [¹⁴C]stearic acid plus [¹⁴C]oleic acid was unchanged by addition of the supernatant fraction and demonstrated that there were no extraction artifacts. Unless otherwise indicated, incubations were performed at 37°C in 1 ml total volume of 0.1 M-potassium phosphate buffer (pH7.4) under air for 15min with initial concentrations as follows: 60 μ M-stearoyl-CoA, 1.2mM- β -NADH and either 0.2mg of protein/ml for microsomes from starved/re-fed rats or 0.7mg of protein/ml for microsomes from control rats. Reactions were stopped by

addition of 1 ml of 10% (w/v) KOH in methanol, and the conversion of stearoyl-CoA to oleoyl-CoA was determined from the ratio of radioactivity recovered as the methyl esters of stearate and oleate, which were prepared and separated by t.l.c. as described previously (Seifried & Gaylor, 1976).

Polarographic assay of stearoyl-CoA desaturase

O₂ uptake was measured at 37°C in a total volume of 3.5 ml. O₂ concentrations were measured with a galvanic oxygen electrode (Mancy *et al.*, 1962) supplied by the Kyushi Kagaku Kenkyusho Co., Tokyo, Japan. The electrode was attached to an offset amplifier for sensitive measurements between 9.0 and 10.0 mV (Gaylor *et al.*, 1975) on a Honeywell Electronik 16 recorder of 1 mV full-scale deflection. O₂ calibrations were carried out either by additions of known volumes of O₂-equilibrated water to the incubation mixture or by the ferricyanide phenylhydrazine method of Misra & Fridovich (1976). Incubations were performed in phosphate buffer as above or in 100 mM-Tris/acetate buffer (pH 7.4 containing 1.0 mM-EDTA). After thermal equilibration of microsomes and buffer, 35 μ l of a 10 mM- α -NADH or 12 mM- β -NADH solution was added, and the increment of O₂ uptake was recorded. A 5.3 mM solution of stearoyl-CoA (40 μ l) was then added, and the additional rate of O₂ consumption was recorded. Reversal of these additions and substitution of oleoyl-CoA for stearoyl-CoA suggested that stimulation of autoxidation by addition of stearoyl-CoA was small relative to the turnover of the desaturase. Inclusion of 0.02 mg of lactate dehydrogenase/ml and 2.0 mM-pyruvate when α -NADH was used produced a 10–12% decrease in oxidation rate due to rapid non-O₂-dependent removal of contaminating β -NADH. When Triton WR-1339 was used to decrease the rate of substrate-independent β -NADH oxidation by microsomes, the detergent (1 mg/ml) was added to the microsomal suspension before additions of β -NADH and stearoyl-CoA (Miyake & Gaylor, 1973).

Nicotinamide nucleotide oxidations

Incubations were performed as described above for O₂ uptake at 37°C (1.02 ml total volume), except that absorbance changes were observed at 340 nm for β -NADH and 344 nm for α -NADH in a Perkin-Elmer model 356 recording spectrophotometer in the dual-beam mode (Miyake & Gaylor, 1973). After addition of NADH, a rapid oxidation change was observed for about 2 min, after which the oxidation rate was constant for over 15 min. Addition of stearoyl-CoA also produced an initial detergent-like clarification, but, after about 15 s, a constant rate of oxidation of NADH was observed. Rates of oxidation were calculated by using $6.22 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ as the absorption coefficient for β -NADH (Rafter &

Colowick, 1957) and $5.6 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ as the absorption coefficient for α -NADH (Okamoto, 1971).

Formation of H₂O₂

Formation of H₂O₂ during desaturation was measured by using scopoletin and horseradish peroxidase by a modification of the method of Thurman *et al.* (1972). Incubations were run as previously described in a total volume of 3 ml with either 50 mM- or 100 mM-NaN₃ to inhibit catalase. Reactions were stopped and NaN₃ was destroyed by placing the samples in a boiling-water bath for 4 min. Precipitated protein was removed by centrifugation. With an Aminco-Bowman fluorimeter, fluorescence emission was measured at 470 nm with 395 nm excitation light. Scopoletin (1–50 μ M) was added and the deflection measured again. Horseradish peroxidase (5 units/ml) was added and the change in fluorescence recorded. Appropriate H₂O₂ standard was added and the fluorescence change also recorded. Scopoletin solutions were standardized by using the absorption coefficient at 345 nm of $12.55 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ (Wellner & Lichtenberg, 1969), and H₂O₂ was standardized by using the absorption coefficient at 230 nm of $61 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Maehly & Chance, 1954). Addition of H₂O₂ standards to incubations showed that less than 10% of internal-standard H₂O₂ was lost during the procedure including the heat-treatment.

Results and Discussion

Incubation of freshly isolated washed control microsomes with increasing amounts of post-micro-

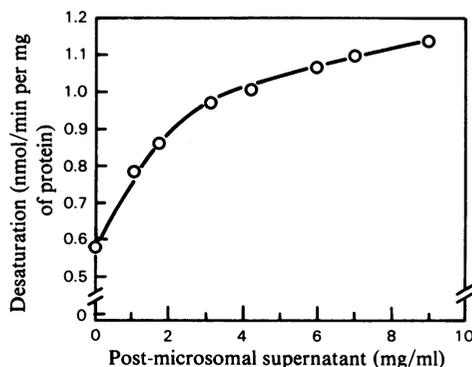


Fig. 1. Stimulation of desaturation by addition of unfractionated post-microsomal supernatant fraction

Incubations were carried out under conditions of initial-velocity measurement (Seifried & Gaylor, 1976) with 60 μ M-substrate, 1 mg of microsomal protein/ml and 5 min of incubation. Data from a single experiment, chosen as representative, are shown.

Table 1. Stimulation of microsomal desaturation by crude post-microsomal supernatant fraction. Incubations were carried out for 15 min with 0.7 mg of microsomal protein/ml and 4 mg of postmicrosomal supernatant protein/ml. Data are given as means \pm S.E.M. with the numbers of samples given in parentheses.

| Microsomes and conditions | Activity (nmol/min per mg of protein) | |
|---------------------------|---------------------------------------|--|
| | -Supernatant fraction | +Supernatant fraction |
| Fresh washed | 0.62 \pm 0.06 (12) | 1.16 \pm 0.04 (12) 0.62 \pm 0.03* (6) |
| Fresh unwashed | 0.75 \pm 0.05 (3) | 0.91 \pm 0.06 (3) |
| Frozen washed | 0.50 \pm 0.02 (7) | 0.51 \pm 0.02 (7) |
| None | — | 0.02 \pm 0.02 (8) |
| After 24 h at 4°C | 0.57 \pm 0.03 (4) | 0.35 \pm 0.10 (4) |

* Supernatant fraction heated to 100°C for 2 min.

somal supernatant fraction (crude cytosol) consistently produced 2-fold stimulation (Fig. 1) of stearoyl-CoA desaturase activity. The stimulatory activity was lost after heat-treatment, but was not lost after dialysis. The extent of stimulation was highly variable, depending upon previous treatment of the microsomes (Table 1). For example, washed microsomes exhibited about the same desaturase specific activity as unwashed microsomes, but activity was enhanced more by addition of the cytosolic fraction to washed than to unwashed microsomes. Presumably, part of the stimulating effect is lost by retention of components of supernatant fraction. Microsomes frozen for more than 2 weeks frequently showed only variable or no stimulation, although a large fraction of initial specific activity was retained. The crude supernatant fraction could not be stored at 4°C. The cytosolic fractions could be frozen and stored at -20°C for 2-4 weeks with only a minor loss of stimulatory activity, and, unlike crude cytosol, after the initial $(\text{NH}_4)_2\text{SO}_4$ fractionation and chromatography

on Sephadex G-100 (see below), stimulatory activity was stable for several days of storage at 4°C. Subsequent experiments were carried out with freshly prepared washed microsomes unless otherwise indicated.

Fractionation of cytosolic proteins

Application of crude cytosol to Sephadex G-100 produced the elution profile shown in Figs. 2(a)-2(d). The three stearoyl-CoA-binding fractions described by Jeffcoat *et al.* (1976) were apparent. These fractions produced inhibition of desaturase at high microsomal protein concentration, but at lower concentrations, stimulation of activity was produced by all three fractions (Fig. 2b). Inhibition was probably due to inhibition by acyl-CoA metabolites, since absorbance at 259.5 nm after acid precipitation of protein from the Sephadex G-100 column revealed three peaks of acyl-CoA content, which coincided well with the binding peaks (Fig. 2a).

Fig. 2. Gel filtration of cytosolic fraction before and after $(\text{NH}_4)_2\text{SO}_4$ fractionation

Samples were filtered on a Sephadex G-100 column (2 cm \times 73 cm), which had been equilibrated with 10 mM-phosphate buffer (pH 7.4 and containing 0.5 M-NaCl) as described by Jeffcoat *et al.* (1976), except that the columns were run at room temperature. (a) and (b) Elution parameters for the crude supernatant fraction; (c) parameters for the protein fraction that precipitated between 40 and 65% saturation with $(\text{NH}_4)_2\text{SO}_4$; (d) the 65-100%-satd. fraction. The amount of protein added was 340 mg for (a) and (b), 180 mg for (c) and 90 mg for (d). $(\text{NH}_4)_2\text{SO}_4$ precipitation was carried out at 0°C by slow addition of crystalline $(\text{NH}_4)_2\text{SO}_4$. The mixture was stirred for 15 min after the final addition, and the precipitate was removed by centrifugation at 10000g for 10 min. Then 5 min before application of samples to the column, [^{14}C]stearoyl-CoA (0.25 μCi) was added, and the fractions were stirred at room temperature. The final concentration of added stearoyl-CoA was about 1 μM . After overnight dialysis of fractions prepared this way, virtually all of the radio-label remained with the protein fraction, which indicates that all of the stearoyl-CoA is protein-bound. Desaturase activities were measured by the radiochemical method with 0.5 ml portions from each fraction/ml of incubation. Concentration-dependence of stimulation was determined only on pooled fractions (see Table 2). Microsomes from starved/re-fed rats (starved for 48 h, re-fed with rat chow 16 h before preparation) were used with either 0.2 mg of protein/ml (b and d) or 2.0 mg of protein/ml (a). CoA-binding fractions were estimated by measuring $A_{259.5}$ of each fraction after removal of protein by precipitation with 5% trichloroacetic acid. —, A_{280} in (a), (c) and (d); \circ , c.p.m./20 μl in (a), (c) and (d); \bullet , A_{259} in (a); \triangle and \blacktriangle , desaturase activity measured with 0.2 and 2.0 mg/ml respectively in (b); \square , desaturase activity in (d).

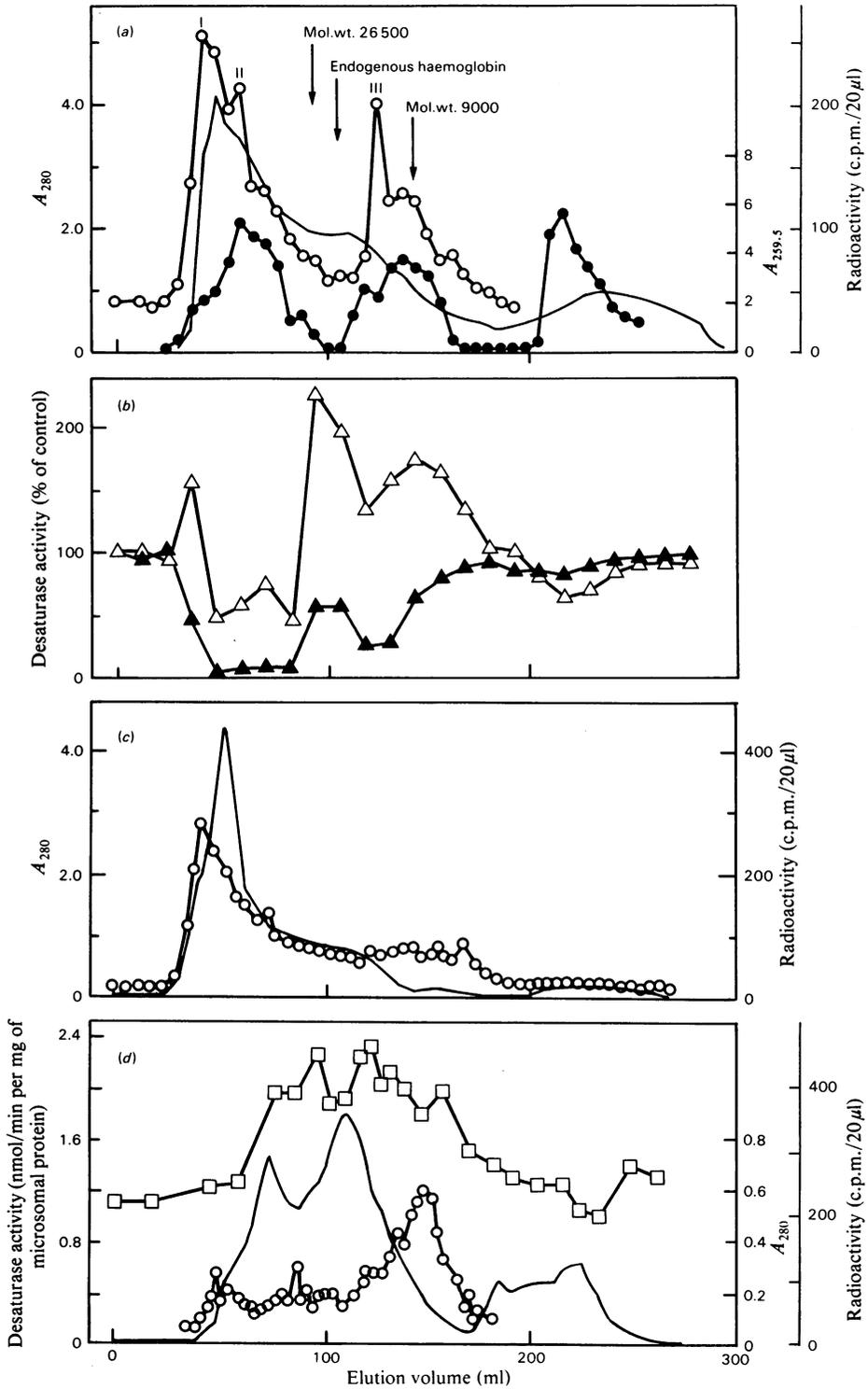


Table 2. *Purification of cytosolic protein*

Data from a representative purification following the scheme described in the text. Specific stimulating activity is determined as half-maximal stimulation of activity divided by the protein concentration required to give half-maximal stimulation with fresh washed control microsomes (0.7 mg of protein/ml). Total stimulating activity is the product of specific stimulating activity and total protein. Yield is expressed as percentage of 65–100%-satd.-(NH₄)₂SO₄ fraction assuming that this fraction contains 100% of the active DEAE-Sephadex fraction. Precipitate from satd. (NH₄)₂SO₄ was solubilized in 10 mM-potassium phosphate (pH 7.4) and dialysed overnight against the same buffer. The dialysed (NH₄)₂SO₄ fraction was adsorbed on to aged calcium phosphate gel (1 ml/10 mg of protein). The solid was collected by centrifugation; the active fraction was recovered by extraction with approx. 30 ml of 0.25 M-phosphate buffer (pH 7.4). The sample was dialysed against 10 mM-phosphate buffer containing 0.5 M-NaCl and fractionated by chromatography on Sephadex G-100 as described in Fig. 2. The active peak from Sephadex G-100 column was dialysed (4°C) against 10 mM-Tris/HCl (pH 8.0 at 20°C) and passed through a PM-30 Diaflo ultrafilter. The sample was then adsorbed to DEAE-Sephadex and eluted as described in Fig. 4. Final activity measurements were for the second active fraction.

| Fraction assayed | Total protein (mg) | Specific stimulating activity (nmol/min per mg of protein) | Total stimulating activity (nmol/min) | Yield (%) | Purification (-fold) |
|---|--------------------|--|---------------------------------------|-----------|----------------------|
| Post-microsomal supernatant | 720 | 0.20 | 145 | — | — |
| 65–100%-satd. (NH ₄) ₂ SO ₄ | 110.5 | 0.27 | 30.2 | 100 | 6.5 |
| Calcium phosphate gel | 48.8 | 0.50 | 24.4 | 80.8 | 12 |
| Sephadex G-100 | 12.6 | 1.50 | 18.9 | 62.6 | 36 |
| PM-30 | 1.10 | 9.99 | 11.0 | 36.4 | 240 |
| DEAE-Sephadex | 0.16 | 46.8 | 7.5 | 24.9 | 1130 |

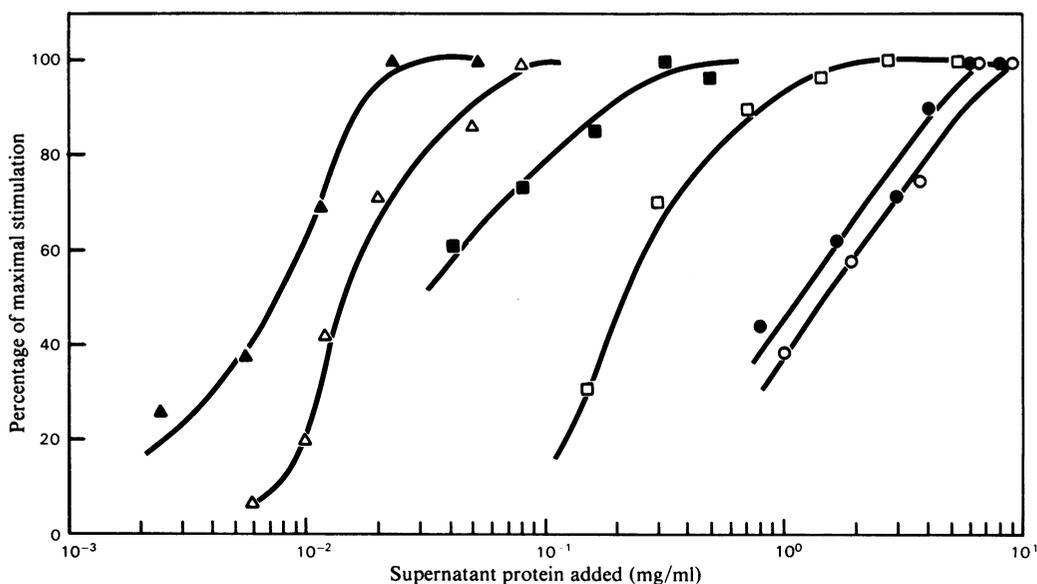


Fig. 3. *Stimulation of desaturase activity by the cytosolic protein(s) at various stages of resolution* (described in Table 2) are crude supernatant (○), 65–100%-satd.-(NH₄)₂SO₄ (●), calcium phosphate gel (□), Sephadex G-100 (■), PM-30 (△) and DEAE-Sephadex (▲). Values are normalized to percentage of maximal stimulation for individual fractions by dividing observed stimulation at specified protein concentrations by maximal stimulation for that fraction. Under all conditions about 100% stimulation could be achieved, suggesting that another component became rate-limiting.

However, unlike these substrate-binding proteins, the most active stimulatory fractions were eluted with proteins of approximate mol.wt. 26500. Further fractionation of the crude cytosolic protein with $(\text{NH}_4)_2\text{SO}_4$ precipitated most of the 26500-dalton stimulatory protein at between 65 and 100% of saturation. Application of the resulting 40–65% and 65–100%-satd. fractions to Sephadex G-100 showed that the stearyl-CoA-binding fraction that was eluted in the void volume was precipitated with the bulk of the protein in the 40–65%-satd. fraction (Fig. 2c). At least three stimulatory fractions were present in the 65–100%-satd. fraction; two were associated with the binding peaks already described by Jeffcoat *et al.* (1976), and a third non-substrate-binding cytosolic protein was eluted just before haemoglobin (Fig. 2d).

Since the latter fraction produced maximal stimulation without binding substrate and thus appeared analogous to the cytosolic protein that stimulates methylsterol oxidase, further purification was carried out. The scheme is summarized in Table 2 and Fig. 3. $(\text{NH}_4)_2\text{SO}_4$ fractionation results in removal of more than 80% of the total protein without an apparent

change in the specific activity of protein that produces stimulation. This is reflected in yield as a loss of more than 80%. However, extensive tests indicated that loss of stimulation appeared to be due primarily to the removal of other stimulatory fractions including the substrate-binding proteins (Fig. 2), and therefore yield ultimately was calculated relative to the recovery of the 65–100%-satd. $(\text{NH}_4)_2\text{SO}_4$ fraction to which different purifications could be related reproducibly. The 65–100%-satd. $(\text{NH}_4)_2\text{SO}_4$ fraction was equilibrated with 10mM-potassium phosphate buffer (pH 7.4) at 4°C by dialysis, and the solution was stirred with a suspension of calcium phosphate gel (10 mg of supernatant protein/ml of gel suspension) for 20 min at 0°C. The gel was collected by centrifugation (3000g for 5 min), and the adsorbed protein was eluted by suspending the resulting pellet in 250mM-potassium phosphate buffer (pH 7.4; 1 mg of gel added/ml) for 20 min before a second centrifugation. The resulting supernatant fraction was decanted from the pellet and dialysed overnight (4°C) against 10mM-potassium phosphate buffer (pH 7.4) containing 500mM-NaCl.

The dialysed solution was fractionated by gel

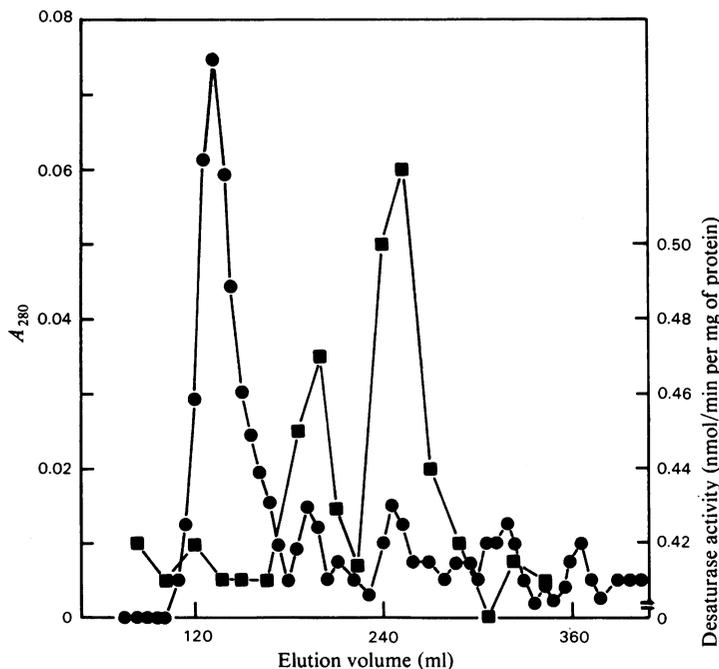


Fig. 4. Fractionation of PM-30 filtrate on DEAE-Sephadex

The clear filtrate from the PM-30 step, which had been equilibrated with 10mM-Tris/HCl buffer (pH 8.0), was applied to a 1.5 cm × 50 cm column and washed with approx. 100 ml of buffer. A linear salt gradient, made with 200 ml of 10mM-Tris/HCl (pH 8.0 and containing 500mM-NaCl), was applied and 6 ml fractions were collected. Stimulation of desaturase activity by the eluted fractions was measured by the radiochemical method by using 0.5 ml of eluate fraction/ml of incubation. ●, A_{280} ; ■, desaturase.

filtration on a Sephadex G-100 column as described above. The active fractions eluted from the calibrated column with a mol.wt. of about 26 500 were combined and dialysed at 4°C overnight against 10mM-Tris/HCl (pH 8.0 at 20°C). The dialysed solution was filtered through a PM-30 membrane (Diaflo ultrafilter; Amicon Corp.) to remove haemoglobin. The resultant clear colourless solution was applied to a DEAE-Sephadex column (1.5cm × 50cm) and eluted with a linear NaCl (0–500mM) gradient.

The elution profile from the DEAE-Sephadex column is shown in Fig. 4. Two peaks that contain stimulatory protein were present; neither was eluted with the major protein peak. The more stimulatory of these fractions, which was eluted second, was associated with a minor protein peak and the other fraction was usually present as a shoulder on the major protein peak. The protein in the second major fraction was therefore further characterized.

Partial characterization of the cytosolic protein

The stimulatory fraction had a u.v.-absorption spectrum characteristic of protein with no unusual properties. No absorption bands were present in the visible range when measured at concentrations up to 0.113mg of protein/ml. Chromatography on a calibrated Sephadex G-100 column confirmed the initial data on the crude fraction that the active fraction was eluted with a mol.wt. of approx. 26 500. Examination by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate revealed a major band (approx. 50% of the total protein) with a mol.wt. of approx. 27 000.

At all stages of purification the extent of stimulation was proportional to protein concentration (Fig. 3). Half-maximum stimulation observed with purified cytosolic protein occurred at 8 µg/ml, and maximal stimulation occurred at about 20 µg/ml when incubated with 0.7 mg of microsomal protein/ml.

The effect of the stimulatory protein on the reaction stoichiometry was examined (Table 3) to determine if the added protein might contain an alternative electron donor or acceptor from that previously recognized for the desaturase reaction. In the first series of experiments α -NADH was used as the electron donor, since microsomal autoxidation with α -NADH is much slower than with β -NADH and thus substrate-independent consumption of O₂ and NADH are much slower. The results showed a 1:1 correspondence between stearoyl-CoA desaturation and O₂ consumption. However, the ratio of α -NADH oxidation to desaturation was greater than 2. The nature of the additional α -NADH oxidation (above that needed for the desaturase reaction) is not known. The rate of α -NADH oxidation showed the same stearoyl-CoA concentration-dependence as the desaturase reaction and therefore may be due to the same enzyme system. The ultimate electron acceptor may be pyruvate, which was added along with lactate dehydrogenase to oxidize contaminating β -NADH. Use of β -NADH as the reductant, which required subtraction of higher autoxidation rates (Table 3), demonstrated that the true reaction stoichiometry is 1 NADH oxidized: 1 O₂ consumed: 1 stearoyl-CoA desaturated. This was unchanged by the addition of the stimulatory protein, which suggests that the protein fraction offers neither an alternative electron donor nor an alternative electron acceptor.

Desaturation as a function of stearoyl-CoA concentration with and without added cytosolic protein is shown in Fig. 5. Over a 10-fold range of concentration the ratio of activities (+/–) remained constant. Thus, in sharp contrast with the acyl-CoA substrate-binding proteins isolated by Jeffcoat *et al.* (1976), the stimulatory protein studied in this report enhanced V_{max} , with no change in K_m (apparent K_m is indicated as K_m' , which seems more appropriate for the expression of substrate concentration-dependence for membrane-bound enzymes).

Table 3. Effect of stimulatory protein on reaction stoichiometry

Washed microsomes were from control rats, and assays were run in 100mM-Tris/acetate (pH 7.4 and containing 1 mM-EDTA). Data for NADH oxidation and O₂ consumption were normalized relative to concurrently determined radiochemical yields. Values are expressed as means ± s.e.m. for the numbers of samples given in parentheses except where only two values are given.

| Assay conducted | | Activity (nmol/min per mg of protein) | |
|--|----------------|---------------------------------------|-----------------------|
| | | –Supernatant fraction | +Supernatant fraction |
| Oleoyl-CoA formation (radioassay) | β -NADH | 0.72 ± 0.03 (4) | 1.18 ± 0.05 (3) |
| | α -NADH | 0.68 ± 0.03 (4) | 1.26 ± 0.04 (4) |
| NADH oxidation (spectrophotometric) | β -NADH | 0.75 ± 0.04 (3) | 1.11 ± 0.05 (3) |
| | α -NADH | 1.46 ± 0.07 (4) | 1.37 ± 0.06 (3) |
| O ₂ consumption (polarographic) | β -NADH | 0.74 ± 0.06 (3) | 1.24 ± 0.10 (3) |
| | α -NADH | 0.68, 0.64 | 1.14, 1.27 |

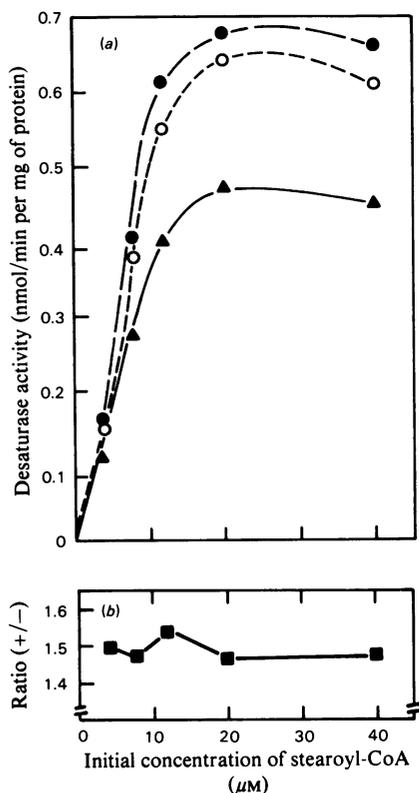


Fig. 5. Effect of cytosolic protein on substrate-dependence of desaturase activity

Desaturation was measured by the radiochemical method. Incubations were carried out for 15 min with microsomes (0.5 mg of protein/ml) from control rats. Cytosolic protein was the combined second stimulatory peak from the DEAE-Sephadex column. No cytosolic protein (▲), 6 μg of cytosolic protein/ml (○), 0.23 mg of cytosolic protein/ml (●), and +/- ratio (■). [The use of ratios of activities (+/-) is a simple test for the effect of supernatant protein on K_m' , and the lack of effect suggests no change in K_m' , which can be readily derived from the Michaelis-Menten equation].

The possibility that stimulation may be due to relief of product inhibition was examined by adding increasing concentrations of oleoyl-CoA to microsomes in the presence and absence of the stimulatory protein. The results showed no effect on K_m' . To examine whether stimulation could be ascribed to removal of oleoyl-CoA for triacylglycerol synthesis (Raja & Reiser, 1972), *sn*-glycerol 3-phosphate was added (Table 4). The results showed inhibition of desaturation, as previously reported (Oshino *et al.*, 1966). These data, along with the presence of stimulation at the earliest time points studied (5 min), suggest that the stimulation is not simply relief of product inhibition.

Since Baker *et al.* (1976) reported stimulation of microsomal desaturation by catalase, we examined the possible role of H_2O_2 production in the desaturase reaction and its possible decomposition by the stimulatory protein. H_2O_2 production by microsomes in the presence of β -NADH can be readily measured if catalase is inhibited with NaN_3 (see the Experimental section). This production was diminished by addition of Triton WR-1339, and under these conditions, simultaneous measurement of desaturation demonstrated that less than 0.1 equiv. of H_2O_2 is produced per desaturase turnover. Alternative experiments were devised to examine whether the stimulation was due to H_2O_2 removal. Incubation of washed fresh microsomes with catalase showed only 13% stimulation of activity, which did not substitute for stimulation by the supernatant protein (Table 4). Incubations with glutathione did not produce stimulation and did not enhance the extent of stimulation produced by the supernatant fraction (Table 4). Direct assay of H_2O_2 dismutation spectrophotometrically (240 nm) by the supernatant fraction showed that it contained no catalase activity, and addition of glutathione did not stimulate H_2O_2 degradation. Therefore the cytosolic protein possesses neither catalase nor glutathione peroxidase activity.

Incubation of the supernatant protein with β -NADH and stearoyl-CoA showed that the protein had no intrinsic desaturase activity (Table 1). Substitution of cytosolic protein for desaturase in the reconstituted system described by Enoch *et al.* (1976), i.e. phosphatidylcholine, cytochrome b_5 , detergent-purified cytochrome b_5 reductase and the cytosolic protein, resulted in an inactive system. Although the stimulatory protein possibly may be a soluble form of desaturase that can utilize only electrons transported via carriers in intact microsomes but not in the solubilized preparations, the large difference in molecular weights (27000 compared with 47000) and the inability to reconstitute the activity argue against this possibility. When supernatant protein was added to a solubilized desaturase system (Gaylor *et al.*, 1970) no stimulation of activity was observed. Similarly, stimulation of the enzymes of methylsterol demethylase by a different cytosolic protein (Gaylor & Delwiche, 1976) has not been observed after solubilization of any of the enzymes from microsomes.

The observation that the cytosolic protein that regulates terminally rate-limiting methylsterol oxidase activity (Williams *et al.*, 1977) also regulates initially rate-limiting hydroxymethylglutaryl-CoA reductase activity (Spence & Gaylor, 1977) may be very important in modulation, in concert, of the microsomal enzymes of cholesterol biosynthesis. The present observations indicate that an analogous protein may also modulate activity of stearoyl-CoA desaturase. Comparison of these proteins reveals that they are not identical (different heat-stability and

Table 4. *Stimulation of microsomal desaturation by second activity peak from DEAE-Sephadex column*
Incubations were run with 0.7 mg of control microsomal protein/ml for 15 min. The amount of added cytosolic protein was less than the amount that produces maximal stimulation, such as shown in Fig. 3, to observe additive effects if present.

| | Activity (nmol/min per mg of protein) | |
|--|---------------------------------------|-----------------|
| | -Peak fraction | +Peak fraction |
| Microsomes + β -NADH | 0.57 \pm 0.03 | 0.85 \pm 0.04 |
| Microsomes - β -NADH | 0.07 | 0.11 |
| Microsomes + β -NADH + glucose + glucose oxidase (0.6 mg/ml) | 0.04 | 0.02 |
| Microsomes + β -NADH + catalase (8 mg/ml) | 0.64 | 0.96 |
| Microsomes + β -NADH + glutathione (10 mM) | 0.53 | 0.84 |
| β -NADH | — | 0.01 |
| β -NADH + phosphatidylcholine (0.1 mg/ml) + cytochrome b_5 reductase (0.13 μ M) + cytochrome b_5 | — | 0.02 |
| Microsomes + β -NADH + <i>sn</i> -glycerol 3-phosphate (6 mM) | 0.27 | 0.22 |

molecular weight, and they are inactive in the counterpart oxidase systems of microsomes), but functionally they may be classified together as non-catalytic regulatory proteins that are probably involved in lipid, sterol and possibly membrane metabolism (Spence & Gaylor, 1977). In addition, certain common properties characterized both proteins. These two proteins have modest to low molecular weights and are present in the post-microsomal supernatant fraction from broken-cell preparations, although the reactions stimulated are catalysed by membrane-bound enzymes. The stimulatory proteins, for both methylsterol oxidase and hydroxymethylglutaryl-CoA reductase, and now for stearoyl-CoA desaturase show hyperbolic saturation curves (Fig. 3). Changes in V_{max} are produced without affecting K_m' . Interestingly, the soluble stimulatory protein of squalene epoxidation exhibits similar hyperbolic saturation, does not bind squalene and apparently affects V_{max} but not K_m' (Tai & Bloch, 1972; Ono & Bloch, 1975). Also, in common with the cytosolic proteins that stimulate desaturase, methylsterol oxidase, squalene epoxidation and hydroxymethylglutaryl-CoA reductase, each protein may necessarily function within membranes, since to date no stimulation has been observed when the membranes have been disrupted by freezing and thawing, by detergents or by reconstitution of catalytic components purified from microsomes.

Additional studies are required to elucidate the mechanism of stimulation by these proteins, to study the action of these proteins in the hydrophobic environment of membranes, and to establish the possible relations in modulation in concert of more than one rate-limiting microsomal enzyme in complex biosynthetic pathways. For example, by analogy to the simultaneous effect of the cytosolic protein on hydroxymethylglutaryl-CoA reductase (Spence &

Gaylor, 1977) and methylsterol oxidase (Gaylor & Delwiche, 1976), the cytosolic protein that stimulates desaturase may also affect either the multienzymic system that produces *de novo* the acyl-CoA substrates for desaturase, i.e. fatty acid synthetase, or the rate-limiting step of oleoyl-CoA utilization. No information on the possible stimulation of hydroxymethylglutaryl-CoA reductase has been reported for the soluble protein that affects squalene metabolism (Tai & Bloch, 1972; Ono & Bloch, 1975; Saat & Bloch, 1976; Ferguson & Bloch, 1977). Thus the reports of several such non-catalytic regulatory proteins suggest a need for further study of a general model of regulation of lipid metabolism at the level of membrane-bound multienzymic systems of the endoplasmic reticulum.

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