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PURIFICATION AND PROPERTIES OF FATTY ACID SYNTHETASE FROM A HUMAN BREAST CELL LINE

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A human mammary epithelial cell line (SKBr3) has been identified in which fatty acid synthetase constitutes up to 28%, by weight of the cytosolic proteins. The enzyme has been purified to near homogeneity from this cell line and some of its properties studied. In common with fatty acid synthetases from other animal tissues, the enzyme is a 480 000 dalton dimer of similar molecular weight subunits, it synthesizes predominantly palmitic acid and is inactive in the absence of free coenzyme A. The kinetic properties and amino acid composition of the enzyme are also similar to those of fatty acid synthetases from various tissues of other animals. Appreciable structural resemblance between human and rodent fatty acid synthetases is indicated by studies on the immunological cross-reactivities of these enzymes.

Introduction

Fatty acid synthetases have been isolated and characterized from a wide variety of animal species and tissues [1]. However, information on the human enzyme is rather limited because of the difficulty in obtaining suitable material for enzyme purification. Fatty acid synthetase has been isolated from the livers of two accident victims [2], but the purified enzyme appears to have been severely nicked, since it could be dissociated into ten polypeptides by SDS-polyacrylamide gel electrophoresis; it is now generally acdepted that animal fatty acid synthetases consist of only two large polyfunctional polypeptides of equal molecular weight [3–6]. In a recent study on the metabolic characteristics of several human breast cell lines we inadvertently discovered an aberrant epithelial cell line which contains prodigious amounts of fatty acid synthetase. These SKBr3 cells provide a convenient starting point for isolation of the human enzyme which is easily obtained in the native, that is 'unnicked', state. Some properties of the human enzyme are reported herein and compared to those of other animal fatty acid synthetases.

Materials and Methods

Culturing of the human breast cell line. The permanent human mammary epithelial cell line, SKBr3 [7], was obtained at the 12th passage from Dr. Martha Stampfer, Peralta Cancer Research Institute, Oakland, CA. Cells were grown to confluency in 175 cm^2 plastic tissue-culture flasks containing a medium consisting of Dulbecco's modified Eagle's minimum essential medium (Gibco, Grand Island, NY), 10% fetal-calf serum (Gibco), 100 units/ml penicillin (Gibco), 100 μg/ml streptomycin (Gibco), 1 μg/ml insulin and 4.5 mg/ml glucose. The medium was changed twice per week. At confluence, cells were detached from five flasks with trypsin and washed four times with Krebs-Henseleit bicarbonate buffer, pH

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7.4/10 mM glucose/1% (w/v) bovine serum albumin/ 0.5 mg/ml trypsin inhibitor and once with 0.25 M sucrose/10 mM dithiothreitol/1 mM EDTA/0.1 mg/ml trypsin inhibitor, pH 7.0. The second large scale preparation was obtained from cells grown on 57 75-cm^2 plastic tissue-culture flasks in the same medium.

Assay of fatty acid synthetase activity. Incubation systems contained 0.1 M potassium phosphate buffer, pH 6.6/0.15 mM NADPH/0.05 mM acetyl-CoA/0.05 mM malonyl-CoA/enzyme in 0.5 ml. Reactions were monitored spectrophotometrically at 340 nm, 37°C. A unit of activity is defined as the amount of enzyme catalyzing the malonyl-CoA-dependent oxidation of 1 nmol NADPH per min.

Molecular weight determination. The molecular weight of the active enzyme was estimated by gel permeation chromatography on a column (160 × 1.5 cm) of Sepharose 6B, using rat mammary fatty acid synthetase (M_r 480 000), trypsinized fatty acid synthetase (M_r 440 000) and catalase (M_r 230 000) as standards. The subunit molecular weight was determined by SDS-polyacrylamide electrophoresis on 5% gels using rat and mouse mammary fatty acid synthetases (M_r 220 000) and trypsinized rat mammary fatty acid synthetase (M_r 125 000 and 95 000) as standards. Gels were stained with Coomassie brillant blue [8].

Amino acid analysis. The sample preparation procedure was identical to that reported previously [9]; a Dionex amino acid analyzer kit was used in conjunction with a column of DC-5A resin, essentially as recommended by the manufacturers. Amino acids were detected using *o*-phthalaldehyde as the fluorogenic reagent. Proline was also assayed fluorimetrically by introduction of a postcolumn reaction with alkaline sodium hypochlorite [10].

Preparation of rabbit anti-fatty acid synthetase immunoglobulins. Purified human fatty acid synthetase (0.5 mg) was injected subcutaneously, in complete Freund's adjuvant, into a young male New Zealand White rabbit. At intervals of three weeks the rabbit was given a subcutaneous booster injection of 0.15–0.3 mg fatty acid synthetase in incomplete Freund's adjuvant. A high titer of antibody was established in two months. Thereafter the rabbit was bled twice weekly. Following each bleeding, the rabbit was given an intramuscular injection of Imferon (50 mg/ml elemental iron, Merrell National Laboratories), 0.1 ml for each 10 ml of blood taken. The immunoglobulin fraction was partially purified by $(NH_4)_2SO_4$ precipitation and chromatography on DEAE-cellulose [11]. Rabbit anti-rat fatty acid synthetase immunoglobulins were also prepared and isolated as described above. Monospecific rabbit anti-mouse fatty acid synthetase immunoglobulins were prepared by affinity chromatography essentially as recommended by Pharmacia, Uppsala, Sweden. All immunoglobulins were stored in 10 mM sodium phosphate buffer, pH 7.2/ 150 mM NaCl at –20°C.

Immunodiffusion studies. 1% agarose gels were prepared in 0.15 M NaCl/0.01 M sodium phosphate buffer (pH 7.2)/5 mM EDTA/0.01 M sodium azide. Diffusion took place at 20–22°C for two days, then gels were washed and subsequently stained with Coomassie brillant blue [7].

The CoA-scavenging system. ATP citrate lyase was used to deplete the fatty acid synthetase assay system of CoA, as described by Sedgwick and Smith [12].

Gas-liquid radiochromatography. Fatty acids synthesized from [2-^{14}C]malonyl-CoA were identified by chromatography of the free acids [13].

Rat, mouse and goose fatty acid synthetases. Fatty acid synthetases were purified from the lactating glands of rats and mice as described previously [14]. Goose uropygial gland fatty acid synthetase [15] was a gift from Drs. A.J. Poulose and P.E. Kolattukudy.

Purification of human fatty acid synthetase. Approx. 1 vol. of packed cells was homogenized, using a Potter-Elvejem homogenizer, in 4–5 vol. 0.25 M sucrose/10 mM dithiothreitol/1 mM EDTA/ 0.1 mg/ml trypsin inhibitor, pH 7.0, and centrifuged at 100 000 × *g* for 1 h. The cytosol was decanted and stirred with solid $(NH_4)_2SO_4$ (114 g/l cytosol) at 0–4°C. The pH was maintained between 6.9 and 7.1 by the addition of KOH. The precipitated proteins were discarded and the supernatant was stirred with additional $(NH_4)_2SO_4$ (129 g/l cytosol). The precipitated proteins were collected by centrifugation and dissolved in 5 mM potassium phosphate buffer, pH 7.0/10 mM dithiothreitol/1 mM EDTA. The volume of the solution was adjusted by dilution so that the conductivity was no greater than that of 0.025 M potassium buffer, pH 7.0. The material was then applied, at 20–22°C, to a column of DEAE-cellulose (bed volume equivalent to 60% of the cytosol vol-

ume), which had been equilibrated previously with 0.025 M potassium phosphate buffer, pH 7.0/10 mM dithiothreitol/1 mM EDTA, and elution with the same solution was continued. The presence of proteins in the column effluent was monitored spectrophotometrically at 280 nm. When all the unadsorbed proteins had been eluted, elution of the adsorbed proteins was commenced using a linear gradient formed from 0.025 M potassium phosphate buffer, pH 7.0/10 mM dithiothreitol/1 mM EDTA and 0.25 M potassium phosphate buffer, pH 7.0/10 mM dithiothreitol/1 mM EDTA (the volume of each buffer used was equivalent to 3–4-times the initial volume of cytosol. Fatty acid synthetase activity emerged from the column when the concentration of potassium phosphate in the eluant was between 0.1 and 0.15 M. Fractions in the center of the elution zone were pooled and stored at 20–22°C overnight prior to fractionation with $(NH_4)_2SO_4$. Proteins were precipitated with $(NH_4)_2SO_4$ (209 g/l), collected by centrifugation, dissolved in a minimum volume of 0.05 M potassium phosphate buffer, pH 7.0/1 mM dithiothreitol/1 mM EDTA and applied to a column (160 × 1.5 cm) of Sepharose 6B, which had been equilibrated previously with 0.25 potassium phosphate buffer, pH 7.0/1 mM dithiothreitol/1 mM EDTA. Proteins were eluted with the same solution and detected spectrophotometrically at 280 nm. Fatty acid synthetase activity eluted with an elution zone maximum at 161 ml (relative elution volume, V_e/V_o = 1.87). Fractions from the center of constant specific activity were pooled and the enzyme was precipitated with $(NH_4)_2SO_4$ (243 g/l). The enzyme preparation was dialyzed against 0.25 M potassium phosphate buffer, pH 7.0/1 mM dithiothreitol/1 mM EDTA. Glycerol was added to 25% and dithiothreitol to 10 mM, final concentration, and the purified enzyme was stored at −70°C.

Results and Discussion

Purification of human fatty acid synthetase. The results of one purification of human fatty acid synthetase are shown in Table I. Homogeneity was confirmed by SDS-polyacrylamide gel electrophoresis (Fig. 1). Our results with two batches of SKBr3 cells indicate that the fatty acid synthetase accounts for 22–28% (w/w) of the cytosolic proteins of the SKBr3 cell. That fatty acid synthetase constitutes a major protein of the SKBr3 cytosol was confirmed by SDS-polyacrylamide gel electrophoresis (Fig. 1).

Molecular weight. The molecular weight of the native human SKBr3 fatty acid synthetase was determined as 480 000 and the subunit molecular weight as 220 000 (Fig. 1), indicating that the enzyme is a dimer of similar molecular weight subunits.

Kinetic properties. Under the standard assay conditions the human SKBr3 fatty acid synthetase synthesized predominantly palmitic acid (90 mol %) with small amounts of myristic and stearic acids. The ability of the enzyme to synthesize butyryl-CoA was not investigated. The pH optimum for the overall reaction of fatty acid synthesis was pH 6.6. The K_m values for substrates were: 18 μM malonyl-CoA, 5 μM acetyl-CoA and 4 μM NADPH. In common with the fatty acid synthetases from the rat liver and mammary gland [12,16], mouse liver and mammary gland [12], and goose uropygial gland (Poulose, A.J., Koluttukudy, P.E., Sedgwick, B. and Smith, S., unpublished

TABLE I

PURIFICATION OF FATTY ACID SYNTHETASE FROM THE HUMAN CELL LINE, SKBr3

A second, large-scale preparation yielded 7 mg fatty acid synthetase from 34 ml cytosol, with an overall recovery of 30%.

Step	Volume (ml)	Protein (mg)	Fatty acid synthetase activity		
			(units)	(units/mg)	(% recovery)
Cytosol	5.1	19.4	5 720	295	100
$(NH_4)_2SO_4$	10	7.4	4 660	630	81
DEAE-cellulose	3.0	2.1	1 580	750	29
Sepharose 6B	14	0.71	960	1 350	17

data) the human SKBr3 fatty acid synthetase was completely inhibited by the CoA-scavenging action of ATP citrate lyase. Enzyme activity was restored on addition of CoA to the CoA-depleted assay system. The mechanism of action of free CoA on the fatty acid synthetase is a controversial issue at present. Linn et al. [18] have suggested that CoA is required for the termination step, whereas work in our laboratory indicates that CoA facilitates the elongation by malonyl moieties of acyl moieties on the fatty acid synthetase [12].

Fig. 1. SDS-polyacrylamide gel electrophoresis of cytosolic and purified fatty acid synthetase proteins from SKBr3 cells. Protein containing six units of fatty acid synthetase, either as cytosol (A) or purified synthetase (B) was electrophoresed on 5% polyacrylamide gels. Human fatty acid synthetase, which bands clearly as the slowest moving component of the cytosolic proteins, was estimated to have a subunit molecular weight of about 220 000.

Structural relationships between human and other fatty acid synthetases. In Table II the amino acid compositions of the human SKBr3 fatty acid synthetase is compared with enzymes from mouse mammary gland (reported for the first time), rat mammary gland [18] and goose uropygial gland [15]. Although the compositions are remarkably similar they provide a rather crude index of structural similarity because of the very large molecular weight of the proteins and the possibility of internal compensation. Immunochemical techniques offer a far more sensitive index for structural comparisons and we found it possible to detect structural differences using the immunodiffusion technique (Fig. 2). Anti-human

TABLE II

AMINO ACID COMPOSITION OF HUMAN FATTY ACID SYNTHETASE; COMPARISON WITH THAT OF OTHER SPECIES

Amino acid	Mol %			
	Human SKBr3	Mouse Mammary	Rat [a] Mammary	Goose [b] Uropygial
Cysteine	1.1	1.7	1.5	2.1
Aspartate	7.6	7.6	7.9	8.7
Threonine	4.6	4.9	5.2	4.4
Serine	7.5	7.7	7.6	7.0
Glutamate	12.2	12.2	10.8	11.2
Glycine	9.3	8.6	8.0	8.2
Alanine	10.5	9.8	8.6	8.0
Valine	7.3	6.6	7.1	7.2
Methionine	1.5	1.7	1.8	1.5
Isoleucine	3.4	3.7	3.6	4.9
Leucine	12.2	11.6	12.2	12.1
Tyrosine	1.1	1.9	2.3	2.5
Phenylalanine	3.1	3.3	3.4	3.2
Histidine	2.7	2.6	2.9	2.8
Lysine	3.5	4.2	4.1	5.7
Arginine	5.5	4.9	5.0	4.3
Proline	5.6	4.9	6.1	4.8
Tryptophan	1.6	2.1	2.1	1.3

[a] From Smith [17].
[b] From Buckner and Kolattukudy [14].

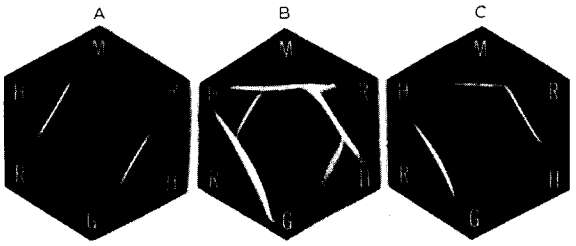

Fig. 2. Immunological cross-reactivity of fatty acid synthetases from human SKBr3 cells, mouse mammary gland, rat mammary gland and goose uropygial gland. Each antibody-antigen combination was run under predeterminined equivalence conditions. Center wells (10 μl) contained the following rabbit anti-fatty acid synthetase immunoglobulins: A, anti-human SKBr3, 9.4 mg/ml; B, anti-mouse mammary, 4.0 mg/ml; C, anti-rat mammary, 31.1 mg/ml. Outer wells (10 μl) contained the following purified fatty acid synthetases: (A) M = mouse mammary, 0.5 mg/ml; R = rat mammary, 0.5 mg/ml; H = human SKBr3, 1.0 mg/ml; G = goose uropygial, 2.3 mg/ml. (B) M = mouse mammary, 4.0 mg/ml; H = human SKBr3, 1 mg/ml; G = goose uropygial, 2.3 mg/ml. (C) M = mouse mammary, 0.5 mg/ml; R = rat mammary, 0.5 mg/ml; H = human SKBr3, 0.5 mg/ml; G = goose uropygial, 0.47 mg/ml.

fatty acid synthetase immunoglobulins, which gave a strong reaction with human SKBr3 fatty acid synthetase, cross-reacted rather weakly with synthetases from rat and mouse mammary glands. A strong spur was formed when human fatty acid synthetase was juxtaposed with either of the two rodent enzymes; the spur always oriented toward the well containing the rodent enzymes. No spur was formed between juxtaposed rat and mouse synthetases. This latter observation indicates that most, if not all the antigenic determinants common to the human and rodent enzymes are present in both the rat and mouse synthetases. Differences in the antigenic determinants in rat and mouse fatty acid synthetases were revealed when antisera raised against either rodent enzyme was used. When juxtaposed rodent enzymes were diffused against anti-mouse fatty acid synthetase antibodies (Fig. 2B), a reaction of partial identity was observed with a spur oriented toward the well containing the rat synthetase. As predicted by the theory of reciprocity, a reaction of partial identity was also observed when juxtaposed rodent synthetases were diffused against anti-rat fatty acid synthetase antibodies (Fig. 2C); the spur was oriented toward the well containing the mouse synthetase. Human fatty acid synthetase gave good immunoprecipitin lines with both anti-mouse (Fig. 2B) and anti-rat (Fig. 2C) fatty acid synthetase antibodies. In both cases the reactions of partial identity were characterized by strong spurs oriented towards the wells containing the human enzyme. The goose uropygial enzyme gave barely visible immunoprecipitin lines with the three immunoglobulin preparations indicating the presence of very few common antigenic determinants on the avian and mammalian enzymes. It has been shown that immunological cross reactivities, as revealed by techniques requiring a multivalent antigen, present a reflection of the degree of sequence resemblance between proteins [19,20]. In general, proteins with sequence differences of 40% or more do not give detectable cross-reactivity. Thus, the results presented here indicate extensive sequence homology between the two rodent enzymes; somewhat less homology between the human and rodent enzymes and relatively poor sequence homology between the mammalian and avian enzymes. These conclusions are consistent with known phylogenetic relationships between these animals.

In conclusion, the human SKBr3 cell line may prove useful as an abundant source of the human fatty acid synthetase enzyme. This enzyme is immunologically identical to the fatty acid synthetase found in normal lactating human breast (Thompson, B. and Smith, S., unpublished data). Thus antibodies raised against the SKBr3 synthetase could be used in an immunoassay to monitor changes in the level of the enzyme during growth and differentiation of human mammary epithelial cells in vitro [21].

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