CELL-FREE SYNTHESIS OF PIGEON LIVER FATTY ACID SYNTHETASE[1]

Carl M. Nepokroeff, Asaf A. Qureshi[2] and John W. Porter

Lipid Metabolism Laboratory, Veterans Administration Hospital,
and the Department of Physiological Chemistry, University of Wisconsin
Madison, Wisconsin 53706

Received September 10,1975

SUMMARY

Pigeon liver fatty acid synthetase proteins (apo- and holo-forms) have been synthesized in a cell-free system reconstituted from polysomes and a soluble enzyme fraction. Identification of the cell-free synthesized products as fatty acid synthetase was achieved by affinity chromatography, by immunoprecipitation and by the simultaneous conversion of both the authentic carrier protein and the in vitro synthesized products from the holo- to the apo-form of the synthetase. The reverse conversion was also effected.

The regulation of fatty acid synthetase is under dietary and hormonal control (1,2). Recent studies have demonstrated that an apo-enzyme exists and the synthesis of this form occurs early during the induction process (3, 4,5). Within 12 hours of the dietary induction period, the maximum rate of synthesis of the apo- and holo-enzyme forms is attained and then the rate of synthesis quickly diminishes (4). Therefore, the regulation of the fatty acid synthetase is an interesting model in which to study the controls that operate at the transcriptional and translational levels.

In order to investigate the macromolecular events in the control of the synthesis of the multi-enzyme complex of fatty acid synthetase, a hepatic cell-free protein synthesizing system, reconstituted from pigeon liver polysomes and a pH 5 enzyme fraction has been employed. In this system in which protein synthesis is programmed by endogenous mRNA, the cell-free synthesis of fatty acid synthetase proteins has been demonstrated by affinity column chromatography, by apo-holo-enzyme protein interconversion and by immunochemical techniques.

---

[1] This investigation was supported in part by grants HL-16364 and AM-01383 from the National Heart and Lung Institute and the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, United States Public Health Service.

[2] On leave from the Pakistan Council of Scientific and Industrial Research, Karachi-39, Pakistan.

MATERIALS AND METHODS

Materials. All experimental chemicals were of reagent grade and [U-^{14}C]leucine was obtained from New England Nuclear. Reagents and procedures for the preparation of the affinity column were previously described (6).

Preparation of pigeon liver polysomes, pH5 enzyme and a high-speed supernatant fraction (S_{100}). The procedures for the preparation of these fractions were as described by Falvy and Staehelin (7) with the following modification. In the isolation of pigeon liver polysomes, the postmitochondrial supernatant of the 33% liver homogenate, containing 0.5% Triton X-100, plus a 25% high-speed supernatant fraction (S_{100}), was layered over a discontinuous sucrose gradient. After ultracentrifugation at 25,000 rpm in a Spinco 30 rotor for 16 hours, the pelleted polysomes were resuspended in buffer without S_{100}. Previous studies had established that the maximum rate of synthesis of the apo- and holo-enzyme forms occurs after a 48-hour fast and a 12-hour period of refeeding (4). Therefore, polysomes were prepared from livers of pigeons which had been refed 12 hours, after fasting, in order to obtain a high level of endogenous mRNA for the fatty acid synthetase. Preparation of the pH 5 enzyme fraction and the high-speed supernatant fraction (S_{100}) were as described previously (7). The S_{100} fraction, used as an RNase inhibitor (8) in the preparation of polysomes, was prepared from rat liver.

Cell-free protein synthesis. A cell-free protein synthesizing system was reconstituted from isolated pigeon liver polysomes and pH 5 enzyme fraction. The incubation conditions of Falvey and Staehelin (7) for protein synthesis were then used. Incubation of the reaction mixture was at 37° for 60 minutes. Large-scale incubation mixtures of 15-20 ml were used for the recovery and analysis of the radioactive protein products synthesized by the cell-free system. 4′-Phosphopantetheine (10 nmoles per ml) was added to insure a source of the cofactor if required in the synthesis of the fatty acid synthetase. The incorporation of [^{14}C]leucine (1.0 μCi/ml) into protein was terminated by addition of carrier [^{12}C]leucine (100 μg/ml), puromycin (2×10^{-4} M) and RNase (2 μg/ml). The reaction mixture was then ultracentrifuged (105,000 × g for 60 min) to prepare the supernatant fraction (S-3) containing the soluble radioactive protein products synthesized *in vitro*. Carrier pigeon liver S-3 fraction was added to this radioactive soluble system as a source of authentic fatty acid synthetase and the mixture was then subjected to the standard procedure for the isolation of fatty acid synthetase (9).

Characterization of the products. A further characterization and identification of the radioactive protein was carried out by the following procedure.

A. Affinity column chromatography. The radioactive products, which co-purified with authentic carrier fatty acid synthetase through the DEAE-cellulose chromatographic step, were subjected to affinity chromatography. The sepharose ε-aminocaproyl pantetheine, used for affinity chromatography, was prepared as previously described (10) and separation of the pigeon liver apo- and holo-enzymes of fatty acid synthetase was achieved by the method described by Qureshi *et al.* (5). Carrier authentic protein was monitored by light absorption at 280 nm and radioactivity was determined by liquid scintillation spectrometry.

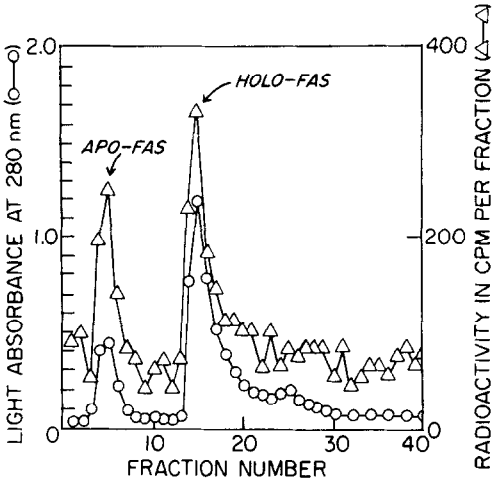

Fig. 1. Affinity chromatography of the DEAE-cellulose purified fatty acid synthetase proteins synthesized by the pigeon liver cell-free system. The fractions eluted from the affinity chromatographic column were assayed for light absorption at 280 nm (o——o) and radioactivity (Δ——Δ). The source of hepatic polysomes was pigeons refed for 12 hours following a 48-hour fast.

B. Apo-holo-enzyme interconversions. Apo- and holo-enzyme fractions were isolated by affinity chromatography, Fig. 1. Each fraction separated on the column contained authentic carrier enzyme protein plus radioactive protein synthesized by the cell-free system. The conversion of the authentic carrier protein and the corresponding radioactive protein from the apo-form to the holo-form and conversely from the holo- to the apo-form was achieved with the following soluble enzyme fractions from pigeon liver.

1. 4′-Phosphopantetheine transferase: conversion of apo- to holo-enzyme. An enzyme fraction, containing 4′-phosphopantetheine transferase activity, which carries out the transfer of 4′-phosphopantetheine from CoA to acyl carrier protein of the apo-enzyme form of the fatty acid synthetase, was prepared as previously described (5). This enzyme was isolated from livers of pigeons that had been refed 12 hours following a 48-hour fast. This enzyme system converts the apo- to the holo-form. Proof of this conversion was established by affinity chromatography.

2. 4′-Phosphopantetheine hydrolase: conversion of holo- to apo-enzyme. An enzyme fraction containing 4′-phosphopantetheine hydrolase activity was prepared from livers of pigeons which had been fasted 16 hours, after a prior 48-hour refeeding period. The hydrolase fraction was prepared according to Qureshi et al. (11). Proof of the holo- to apo- conversion was established by affinity chromatography.

C. Immunochemical precipitation of the radioactive fatty acid synthetase species. Immunochemical precipitation was carried out on the radioactive holo-enzyme purified by affinity chromatography. The procedure followed was reported previously (4). In all reactions an excess of antiserum was used and corrections for nonspecific coprecipitation were made by the

second challenge method. The immunoprecipitates were washed with 1% Triton X-100 and carrier leucine to reduce nonspecific binding and precipitation.

RESULTS

The in vitro synthesis of protein by the reconstituted pigeon liver cell-free system requires both polysomes and the pH 5 enzyme fraction. In the absence of these fractions activity was 9% and 19%, respectively, of that of the complete system. Protein synthesis in the complete system was effectively blocked by puromycin and cyclohexamide (0.2 mM) and by RNase (10 μg/ml). It is probable that the synthesis of fatty acid synthetase observed in the pigeon liver system is due to chain elongation, since pyrocatechol violet and aurintricarboxylic acid (0.2 mM), which are inhibitors of protein chain initiation (12), had no effect on the total incorporation but blocked synthesis directed by brome mosaic virus RNA in a wheat embryo cell-free system.

The identification of fatty acid synthetase in the [^{14}C]leucine-labeled products was achieved through the use of affinity column chromatography (Fig. 1). The radioactive proteins synthesized in vitro cochromatographed with the authentic carrier apo- and holo-fatty acid synthetases. This result is evidence that the fatty acid synthetase is synthesized in the reconsituted pigeon liver cell-free system.

Additional proof of the in vitro synthesis of the fatty acid synthetase was obtained through the conversion of radioactive apo- to holo-enzyme and the conversion of radioactive holo-enzyme to the apo-form with the appropriate crude enzyme fractions, 4′-phosphopantetheine transferase and hydrolase, respectively. The coconversion of the authentic enzyme form and the radioactive synthesized product to the alternate enzyme form is shown in Table I. Treatment of the apo-enzyme, purified by affinity chromatography, with a 4′-phosphopantetheine transferase enzyme fraction resulted in the formation of holo-enzyme. The conversion of mass and radioactivity was about 47%; i.e., 4.0 mg and 2260 cpm of the apo-form to 1.9 mg and 1115 cpm of holo-enzyme. The reciprocal conversion, holo- to apo-enzyme, is also shown in Table I. Since the 4′-phosphopantetheine hydrolase enzyme fraction from pigeon liver also contains a kinase which converts holo-a- to holo-b-fatty acid synthetase (11), incubation of the holo-a-enzyme with this fraction resulted in the formation of both apo- and holo-b-enzyme. Conversion of the holo-a-enzyme to the apo- and holo-b-enzymes was about 63%.

TABLE I

CO-INTERCONVERSION[a] OF AUTHENTIC FATTY ACID SYNTHETASE ENZYME FORMS AND THE IN VITRO SYNTHESIZED FATTY ACID SYNTHETASE PROTEINS

Enzyme form	Conversion of apo- to holo-enzyme			
	Initial reactant		Conversion products	
	Protein mg	Total cpm	Protein mg	Total cpm
Apo-enzyme[b]	4.0	2260	1.5	750
Holo-enzyme[b]	-	-	1.9	1115
	Conversion of holo- to apo-enzyme			
	Initial reactant		Conversion products	
	Protein mg	Total cpm	Protein mg	Total cpm
Holo-a-enzyme	6.0	3008	1.9	716
Holo-b-enzyme	-	-	2.7	1123
Apo-enzyme	-	-	1.1	673

[a]The term "co-interconversion" means the conversion of both authentic protein mass and radioactive protein of one enzyme form to the other. Authen tic carrier protein is reported in mass (mg); in vitro synthesized enzyme is given in radioactivity (cpm).

[b]The reactants, apo- and holo-a-fatty acid synthetases were isolated by affinity chromatography. The products formed from these substrates were also identified and isolated by the affinity chromatographic method. Holo-enzyme consists of a mixture of the holo-a and holo-b forms of the pigeon liver fatty acid synthetase. The identification and isolation of these forms has been reported by Qureshi et al. (11). The apo-enzyme formed in the interconversion of holo-a- to apo-enzyme was further purified by DEAE-cellulose chromatography after affinity chromatography.

Immunoprecipitation was used to provide confirming evidence of the synthesis of the fatty acid synthetase species. Radioactive holo-enzyme, isolated by affinity chromatography, was challenged with antisera specific for the pigeon or for the rat liver fatty acid synthetase. Rat liver fatty acid synthetase was added as carrier antigen to incubations with rat liver antiserum, since rat and pigeon liver anti-fatty acid synthetase serums do not cross-react. Antiserum, monospecific for pigeon liver fatty acid synthetase, pre-

TABLE II

CHARACTERIZATION OF THE RADIOACTIVE CELL-FREE PRODUCTS

[^{14}C]Leucine-labeled protein fraction	Total radioactivity cpm incorporated
Total protein	8×10^5
Released proteins (S-3 fraction)	3×10^5
DEAE-cellulose purified fraction	3×10^3
Affinity column chromatography (holo- plus apo-fatty acid synthetase)	1×10^3
Immunoprecipitable radioactivity	
Precipitate formed with pigeon liver fatty acid synthetase antiserum	600
Precipitate formed with rat liver fatty acid synthetase antiserum	10
Control serum	0

The total radioactive protein fraction (8×10^5 cpm) was obtained from two pooled, typical 15-ml cell-free incubations. Immunoprecipitation was then performed on the holo-fatty acid synthetase isolated by affinity chromatography. A total of 700 cpm of holo-enzyme was used for immunoprecipitation. The pigeon liver fatty acid synthetase antiserum precipitated 86% (i.e., 600 cpm) of this radioactivity.

cipitated almost all of the radioactive protein (85%) which copurified with the holo-enzyme form on affinity chromatography, Table II. Neither control serum nor antiserum specific for the rat liver enzyme precipitated a significant amount of radioactive protein.

The low incorporation of radioactivity into the fatty acid synthetase, Table II, illustrates a general problem in the cell-free synthesis of hepatic protein. Approximately 0.33% of the radioactive protein released from the polysomes was fatty acid synthetase. Similar problems of poor specific synthesis and chain release have been reported for the in vitro synthesis of tryptophan oxygenase programmed by purified rat liver mRNA (13).

DISCUSSION

The demonstration of the synthesis of pigeon liver fatty acid synthetase

by a cell-free amino acid incorporating system has been presented in this paper. Proof of the in vitro synthesis of the pigeon liver fatty acid synthetase has been provided by affinity chromatography of DEAE-cellulose purified fatty acid synthetase, by the enzymatic interconversion of the apo- and holo-enzyme forms of authentic plus radioactive enzyme and by immunoprecipitation with monospecific antiserum.

The reconstituted pigeon liver cell-free system synthesizes both the apo-enzyme and the holo-enzyme forms of the fatty acid synthetase (Fig. 1). The in vitro synthesis of these protein products appears to result from the completion of nascent polypeptide chains and not from de novo synthesis. Since the level of in vitro synthesis of the fatty acid synthetase is low, and since the level of initiation may be negligible in this reconstituted polysome cell-free system, further studies with this system may have limited applicability to the study of translational and transcriptional control. However, these studies do suggest that the isolation of biologically active mRNA coding for the fatty acid synthetase may be feasible. The isolation of specific mRNA and its translation in a cell-free protein-synthesizing system would of course permit quantitative studies of changes in the level of mRNA in the control of the fatty acid synthetase. Using methods which quantitate specific mRNA (13), it should be possible to elucidate the macromolecular events for the regulation of the fatty acid synthetase in normal and malignant tissue.

It has been established that the molecular weight of the two nonidentical half-molecular weight subunits of pigeon liver fatty acid synthetase complex is approximately 240,000 (14). Recently it has been suggested that each of the subunits may be a single, large polypeptide (15). If so, then the mRNAs coding for these proteins and the polysomes containing the synthetase mRNA would have unique size properties which should favor their isolation by affinity chromatography and gel filtration methods. Studies in our laboratory are in progress to isolate the specific mRNA for the synthetase and to test the fidelity of its translation.

ADDENDUM

During the course of these investigations, A. W. Alberts et al. reported preliminary studies on the synthesis of rat liver fatty acid synthetase at the 59th Annual Meeting of the Federation of American Societies for Experimental Biology.

REFERENCES

1. Nepokroeff, C.M., Lakshmanan, M.R., Ness, G.C., Muesing, R.A., Kleinsek, D.A. and Porter, J.W. (1974) Arch. Biochem. Biophys. 162, 340-344.
2. Lakshmanan, M.R., Nepokroeff, C.M. and Porter, J.W. (1972) Proc. Nat. Acad. Sci. USA 69, 3516-3519.
3. Yu, Hon Lun and Burton, D.N. (1974) Arch. Biochem. Biophys. 16, 297-305.
4. Lakshmanan, M.R., Nepokroeff, C.M., Kim, M. and Porter, J.W. (1975) Arch. Biochem. Biophys. In press.
5. Qureshi, A.A., Kim, M., Lornitzo, F.A., Jenik, R.A. and Porter, J. W. (1975) Biochem. Biophys. Res. Commun. 64, 836-844.
6. Lornitzo, F.A., Qureshi, A.A. and Porter, J.W. (1974) J. Biol. Chem. 249, 4732-4744.
7. Falvey, A.K. and Staehelin, T. (1970) J. Mol. Biol. 53, 1-19.
8. Blobel, G. and Potter, V.R. (1967) J. Mol. Biol. 28, 539-542.
9. Muesing, R.A. and Porter, J.W. (1975) in Methods in Enzymology, J. M. Lowenstein, Ed., Vol. XXXV, pp. 45-49. Academic Press, New York.
10. Lornitzo, F.A., Qureshi, A.A. and Porter, J.W. (1974) J. Biol. Chem. 249, 1654-1656.
11. Qureshi, A.A., Jenik, R.A., Kim, M., Lornitzo, F.A. and Porter, J. W. (1975) Biochem. Biophys. Res. Commun. In press.
12. Huang, M.T. and Grollman, A.P. (1973) Biochem. Biophys. Res. Commun. 53, 1049-1059.
13. Schutz, G., Kellewich, L., Chen, G. and Feigelson, P. (1975) Proc. Nat. Acad. Sci. USA 72, 1017-1020.
14. Kumar, S., Muesing, R.A. and Porter, J.W. (1972) J. Biol. Chem. 247, 4749-4762.
15. Stoops, J.K., Arslanian, M.J., Oh, Y.H., Aune, K.C., Vanaman, T.C. and Wakil, S.J. (1975) Proc. Nat. Acad. Sci. USA 72, 1940-1944.