A Novel Procedure for the Preparation and Characterization of Catalytically Active Fatty Acid Synthetase Immobilized on Sepharose Beads*

(Received for publication, April 25, 1986)

Jan Mikkelsen‡, Jens Knudsen‡, and Stuart Smith‡

From the Research Laboratory, Children's Hospital Medical Center, Oakland, California 94609 and the §Institute of Biochemistry, Odense University, Odense, Denmark

A novel procedure for immobilization of enzymatically active fatty acid synthetase is presented. The enzyme is coupled to a Sepharose 4B matrix containing covalently attached antibodies which recognize, and bind specifically to, the thioesterase domain of this polyfunctional enzyme. A continuous flow system is described for assay of the immobilized enzyme. Fatty acid synthetase activity apparently is not limited by movement of substrates through the Nernst diffusion layer surrounding the matrix particles, since normal Michaelis-Menten kinetics are observed and reaction rates are independent of flow rate. The $K_m$ values for acetyl-CoA and malonyl-CoA, the pH/activity profile, and the reaction products are essentially the same as for the freely soluble enzyme, although the specific activity is lower by about 55%.

The preparation and characterization of immobilized subunits of the enzyme could provide a valuable approach for studying the role of structural and functional subunit interactions in the enzyme. In addition, the immobilized enzyme offers a model for studying the properties of this enzyme in a highly structured environment such as might exist in vivo, permitting study of both physical and functional interactions of fatty acid synthetase with other lipogenic enzymes.

Enzymes of the de novo fatty acid biosynthetic pathway, such as acetyl-CoA carboxylase, fatty acid synthetase, and ATP citrate lyase, usually are isolated from the cytosol of animal cells as soluble proteins. However, in vivo, these enzymes probably function within the framework of a highly organized structure, either in an environment resembling a gel, or adsorbed to membrane interfaces, or in a supramolecular complex. The possibility that some of the lipogenic enzymes might be structured into a supramolecular complex organized on either endoplasmic reticulum membranes or on a matrix of polymeric acetyl-CoA carboxylase filaments has already been raised by others (1-3). The immobilized enzyme may provide a model system that approximates more closely the perinuclear lipid droplet, permitting study of both physical and functional interactions of fatty acid synthetase with other lipogenic enzymes.

*This investigation was supported in part by Grant AM 16073 from the National Institutes of Health, United States Public Health Service. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Present address: Institute of Biochemistry, University of Odense, Odense, Denmark.

§ To whom correspondence should be addressed: Research Laboratory, Children's Hospital Medical Center, 747 52nd St., Oakland, CA 94609.

1 A. Stern and S. Smith, unpublished results.
established by monitoring the appearance of the column effluent using a flow cell, spectrophotometer, and recorder. Syringe B, attached to the substrate-delivery manifold system, was used to draw liquid into the tubing leading from each substrate container to its individual valve. The enzyme reaction was initiated by closing the valve leading to reservoir B and opening valve 1 of the substrate delivery manifold. This operation allowed transport of the substrates malonyl-CoA and acetyl-CoA, dissolved in 0.2 M potassium phosphate buffer (pH 6.8), 0.1 mM dithiothreitol, from reservoir A occurred, to the immobilized enzyme. Once steady state conditions had been established, as evidenced by a lower, stable absorbance at 254 nm, valve 1 was closed and valve 2 opened. This operation allowed establishment of new steady reaction conditions depending on the composition of the substrate mix in the second tube. Sequential introduction of various substrate mixes from the seven ports of the manifold facilitated rapid determination of optimum substrate concentration, optimum pH, etc. When required, the effluent from the column was collected for estimation of flow rate and for product analysis.

CoA, malonyl-CoA, butyryl-CoA, acetoacetyl-CoA, 3-hydroxybutyryl-CoA, and crotonyl-CoA, obtained from Sigma, were used as standards.

Fatty Acid Products—Sepharose 4B gel containing the radioactive reaction products was heated at 90°C for 45 min in 1.0 M ROH. The mixture was acidified with HCl, carrier fatty acids were added, and the fatty acids extracted by partitioning into petroleum ether. Radioactivity was assayed on a portion of the extract, by liquid scintillation spectrometry, and the remaining fatty acid was methylated with boron trifluoride/methanol (9) for analysis by gas-liquid radiochromatography (10).

RESULTS AND DISCUSSION

The amount of enzyme immobilized per unit volume of gel was estimated radiochemically using 2-14C-labeled carboxamidomethylated fatty acid synthetase. Two separate experiments yielded identical results, 78 µg of enzyme immobilized per milliliter of gel. Enzymatic activity of several preparations of fatty acid synthetase was assessed using a continuous flow system. The extent of substrate utilization could be controlled by varying either the amount of enzyme in the column or the flow rate of substrates through the column. The specific activity of the immobilized enzyme was 772 ± 88 (n = 3) units/mg. The same enzyme preparations, when assayed in the soluble form, gave a specific activity of 1782 ± 115 (n = 3) units/mg. The immobilized enzyme column was stable for at least 1 day; the effect of prolonged storage was not examined.

Theoretical considerations have led to predictions that the kinetics for an immobilized enzyme should differ substantially from those for an enzyme in free solution (11-15). The theory predicts that for an enzyme immobilized in a continuous flow system, at low substrate concentrations or low flow rates the reaction will be diffusion-controlled to some extent. The theory has been supported by experimental work with a number of immobilized enzymes including alkaline phosphatase (15), acetylcholinesterase (16), and chymotrypsin (17). However, under our conditions of assay, the amount of NADPH oxidized by the fatty acid synthetase was directly proportional to the reciprocal of the flow rate, i.e. the extent of substrate utilization was directly proportional to the time.

**Carboxamidomethylation**

Rat liver fatty acid synthetase, dissolved in 0.1 M potassium phosphate buffer (pH 6.8) was incubated for 30 min, in the dark at 20 °C, with iodo[2,14C]acetamide (69 Ci/mol), 5 mol/mol of enzyme. The enzyme was precipitated twice with 50% saturated ammonium sulfate and dialyzed against 0.2 M potassium phosphate buffer (pH 6.9), 10 mM dithiothreitol. Prepared under these conditions, the fatty acid synthetase contained 4.2 mol of carboxamidomethyl moieties/mol of enzyme dimer. This radiolabeled enzyme was used to estimate the amount of fatty acid synthetase bound to the antibody column.

**Product Analysis**

In these experiments, [2,14C]malonyl-CoA (7-12 Ci/mol) replaced unlabeled malonyl-CoA.

Acyl-CoA Products—CoA thioesters emerging in the eluent from a Sepharose 4B fatty acid synthetase column were identified quantitatively by high pressure liquid chromatography (8). Authentic acetyl-CoA, butyryl-CoA, 3-hydroxybutyryl-CoA, and crotonyl-CoA were introduced with the buffer. The flow rate was controlled with a peristaltic pump. B, plot of NADPH oxidation as a function of the reciprocal of flow rate. Data points fit the straight line with a correlation coefficient of 0.997.

**Fig. 1. Continuous flow system for assay of immobilized fatty acid synthetase**

**Fig. 2. Effect of flow rate on activity of immobilized fatty acid synthetase**. Reaction mixtures containing acetyl-CoA (35 µM), malonyl-CoA (70 µM), and NADPH (100 µM) were pumped, at various flow rates, through a column (0.24-mI bed volume) of Sepharose 4B containing 18.7 µg of immobilized fatty acid synthetase. A, recording of output from spectrophotometric flow cell. Initial baseline was established by pumping buffer and NADPH only through the column. At the point indicated by the arrow, acetyl-CoA and malonyl-CoA were introduced with the buffer. The flow rate was controlled with a peristaltic pump. B, plot of NADPH oxidation as a function of the reciprocal of flow rate. Data points fit the straight line with a correlation coefficient of 0.997.
of exposure of enzyme to substrate (Fig. 2). Thus, the reaction rate was independent of flow rate. The conclusion that the rate of fatty acid synthesis was not limited by diffusion of substrates was further supported by studies on the effect of substrate concentration. Classical Michaelis-Menten kinetics were observed with both acetyl-CoA and malonyl-CoA (Fig. 3). Were diffusion control to be operative at low substrate concentrations, the Lineweaver-Burk plot would be convex to the 1/[S] axis (18). The measured K_m values for acetyl-CoA (5 μM) and malonyl-CoA (13 μM) were similar to values obtained with the freely soluble enzyme under standard assay conditions: acetyl-CoA, 3 μM (19) and malonyl-CoA, 13 μM (20). The pH/activity profile of the immobilized enzyme was not noticeably different from that of the freely soluble enzyme; both forms of the enzyme exhibit a pH optimum at 6.6 (Fig. 4).

The apparent lack of effect of flow rate on the specific activity of the fatty acid synthetase and the observance of normal Michaelis-Menten kinetics over a wide range of substrate concentrations indicate that movement of substrates across Nernst diffusion layer surrounding the gel surface does not limit the overall reaction rate. Diffusion-independent conditions might be anticipated when the diffusion coefficients for the substrates are high or the rate of catalysis is low. A high diffusion coefficient will allow substrate rapid access to the immobilized enzyme, whereas a “slow” reaction would imply that the catalytic reaction itself, rather than the diffusion process, is rate-limiting. Most enzymes which have been found to be diffusion controlled in the immobilized state have V_max much greater than fatty acid synthetase (for example, acetylcholinesterase, 0.3 mmol/min/mg (16); catalase, 2 mmol/min/mg (14); and glucose oxidase, 0.1 mmol/min/mg (21)). The immobilized forms of these enzymes also exhibit altered activity/pH profiles; although the optimum pH values are usually unchanged, the reactions appear to be less pH-dependent than with the freely soluble form of the enzymes. These data can be interpreted to indicate that diffusion constraints depend not only on the velocity of substrate entry but also on the rate of the enzymic catalysis. Since greater rates of catalysis will impose greater diffusion constraints, any factor, such as suboptimal pH, that decreases the catalytic rate will reduce the diffusion constraint.

The products of synthesis by the immobilized enzyme were similar to those of the freely soluble enzyme (Table I). Most of the NADPH consumed (95%) was utilized to support the synthesis of long-chain fatty acids; the remainder accounted for the synthesis of butyryl-CoA and acetoacetyl-CoA.

Acetoacetyl-CoA, β-hydroxybutyryl-CoA, crotonyl-CoA, and butyryl-CoA are all good substrates for the rat fatty acid

---

**FIG. 4. Effect of pH on activity of immobilized fatty acid synthetase.** Reaction mixtures containing acetyl-CoA (60 μM), malonyl-CoA (60 μM), and NADPH (102 μM), in potassium phosphate buffer of various pH, were pumped, at 0.54 ml/min, through a column (bed volume, 0.15 ml) of Sepharose 4B containing immobilized fatty acid synthetase. Activity was recorded from spectrophotometric flow cell. Initial baseline was established by pumping buffer and NADPH only. At the point indicated by the arrow, acetyl-CoA and malonyl-CoA were introduced with the buffer. B, plot of NADPH oxidation rate vs pH. Data points represent the average of two experiments ± range. The dotted line indicates the activity/pH profile for freely soluble enzyme (from Ref. 10).
TABLE I

Products formed by immobilized fatty acid synthetase

<table>
<thead>
<tr>
<th>Product</th>
<th>Rate of synthesis (nmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADP</td>
<td>968</td>
</tr>
<tr>
<td>Butyryl-CoA</td>
<td>14</td>
</tr>
<tr>
<td>Acetoacetyl-CoA</td>
<td>7</td>
</tr>
<tr>
<td>(\beta)-Hydroxybutyryl-CoA</td>
<td>0</td>
</tr>
<tr>
<td>Crotonyl-CoA</td>
<td>0</td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>42</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>15</td>
</tr>
</tbody>
</table>

The antibody-antigen complex. Indeed, the intricate spatial relationships between the functional domains of the synthetase is preserved, and the immobilized enzyme retains the ability to coordinate the functions of its constituent domains in catalyzing the series of reactions leading to the formation of fatty acids. The immobilized, catalytically active fatty acid synthetase offers a unique model for studying intersubunit communication as well as physical and functional interactions of the synthetase with other lipogenic enzymes. An example of the application of this model system is presented in the accompanying paper.

Acknowledgment—We thank Alan Stern for preparing the enzyme and antibody used in this study.

REFERENCES