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Photolabeling acyl CoA binding proteins in microsome preparations

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Abstract To identify key enzymes that participate in acylglycerol metabolism, 12-[(5-iodo-4-azidosalicyl)amino]dodecanoyl-coenzyme A (ASACoA) was employed as a photoaffinity label for those enzymes that use fatty acyl CoA as a co-substrate. ASACoA inhibited diacylglycerol acyltransferase activity in liver microsomes and was incorporated into triacylglycerol in a microsome dependent reaction. When photoactivated, ASACoA labeled four proteins in rat liver (75, 54, 50 and 37 kDa) and in epididymal fat cell (75, 64, 54 and 37 kDa) microsomes. Photolabeling was sensitive to palmitoyl CoA. After solubilization in Triton X-114, all four proteins were concentrated into the detergent phase, indicating that they are integral membrane proteins.

Key words: Photolabeling; Diacylglycerol acyltransferase; Microsome; Detergent

1. Introduction

The enzymes in lipid biosynthetic pathways are significant in that they regulate the production and turnover of recently discovered lipid second messengers as well as account for the accumulation of structural and storage lipid. Two of these enzymes that have resisted attempts to achieve their isolation and characterization are monoacylglycerol acyltransferase (EC 2.3.1.22; MGAT) and diacylglycerol acyltransferase (EC 2.3.1.20; DGAT). MGAT catalyzes the synthesis of *sn*-1,2-diacylglycerol from *sn*-2-monoacylglycerol and fatty acyl CoA. This enzyme has been recently partially purified from neonatal rat liver although its activity could not be identified as a specific protein on polyacrylamide gels under denaturing conditions [1]. DGAT is the terminal enzyme in triacylglycerol synthesis. Its substrates include fatty acyl CoA and diacylglycerol, a second messenger and important branch point intermediate in acylglycerol metabolism. DGAT is found only in eukaryotes, and in animals is expressed primarily in the mammary gland, liver, adipose tissue, and the intestinal epithelium [2]. The active site of this microsomal enzyme faces the cytosol [3] and its size was estimated at 72 kDa from radiation inactivation analysis [4]. DGAT activity was reportedly enriched 415-fold from washed rat liver microsomes by immunoaffinity chromatography employing a monoclonal antibody prepared against a crude microsomal membrane extract [5]. The purified protein fraction exhibited major bands of 60 and 77 kDa upon denaturing gel electrophoresis in the presence of reducing agents. It was unclear whether the activity corresponded to one of these bands or a minor component in the preparation. A large complex of several acylglycerol synthetic enzymes including both MGAT and DGAT was isolated from rat intestinal mucosa [6]. This preparation showed a number of silver-stained bands between 52 and 72 kDa. The inability of direct biochemical fractionation to identify specific isolated protein band with activity for either fatty acyltransferase suggests that alternative methods should be employed to achieve this same goal.

Photoaffinity labeling represents an alternative approach to identify proteins catalyzing reactions in the pathway leading to synthesis of triacylglycerol. The radiolabeled fatty acyl CoA analogue, 12-[(5-iodo-4-azidosalicyl)amino]dodecanoyl-coenzyme A (ASA-CoA), and similar photoactivable analogues of fatty acyl CoA have been employed in a number of studies to photolabel proteins present in plant microsomes [7,8] and the isolated enzymes acyl-CoA: glycine *N*-acyltransferase [9], transcarboxylase [10], pyruvate carboxylase [11], acylCoA: cholesterol-acyltransferase [12], and cholesterol esterase [13]. Although a number of mammalian enzymes use fatty acyl CoA as a substrate or regulatory molecule, photolabeling of stripped liver and fat cell microsomes, where MGAT and DGAT activities are concentrated, might selectively identify one or more of these acyltransferase enzymes. DGAT, in particular, has a broad substrate specificity for fatty acyl CoAs [2] and thus may be amenable to photolabeling. Identified proteins could then be sequenced so that probes could be designed to identify cDNA's coding for DGAT from an appropriate library.

Here I identify a few, discrete protein bands that are labeled by [^{125}I]ASACoA in microsomes from two tissues active in the synthesis of triacylglycerol. These labeled proteins appear to be integral membrane proteins that exhibit selective interaction with the fatty acyl CoA analogue.

2. Experimental procedures

2.1. Synthesis of ASACoA

The photolabeling reagent was synthesized essentially as described [7] with all steps being carried out in a darkened room. The unlabeled product (43% yield based on CoA) was concentrated to 0.5 ml and 0.75 mCi carrier-free [^{125}I]NaI (New England Nuclear) in 7.5 μl and 60 μl chloramine-T (Sigma) in acetone (10 mg/ml) added and kept at room temperature for 2 h. The labeled ASACoA was purified by preparative thin layer chromatography on 1 mm Silica gel G plates in butanol/water/acetic acid (50:30:20, v/v/v, R_f = 0.46), eluted in absolute methanol, and kept at −20° until used; specific activity = 2.5 Ci/mmol.

2.2. Preparation of microsomes

Liver microsomes were prepared from a single male rat as described [5]. Isolated microsomes were treated with 1 mM deoxycholate and washed to remove luminal and loosely bound protein and stored in 0.05 M Tris-HCl (pH 7.8), 300 mM sucrose, 1.25 mM EDTA at a concentration of 11 mg protein/ml. Rat epididymal fat cells and microsomes were prepared as described [14] and stored at −70°C at a concentration of 4.3 mg protein/ml.

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Abbreviations: ASACoA, 12-(5-iodo-4-azido-2-salicyl)amino]-dodecanoyl-coenzyme A; DGAT, diacylglycerol-acyltransferase; MGAT, monoaclyglycerol-acyltransferase; SDS, sodium dodecyl sulfate; CoA, coenzyme A.

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2.3. Photolabeling and separation of microsomal proteins

For each photolabeling to be performed, 10 μl (0.4 nmol) [^{125}I]ASACoA was evaporated in a 1.5 ml Eppendorf centrifuge tube under a stream of nitrogen. To each tube was added 0.05 ml of 0.085 M Tris-HCl (pH 8.0), 4 mM $MgCl_2$ and the tube vortexed to solubilize the ASACoA. 0.02 ml of rat liver microsomes or 0.05 of fat cell microsomes was then added, the suspension made to 0.1 ml with water and maintained at 0°C in the dark. After half-an-hour, the reaction mix was transferred to the cap of an Eppendorf tube, covered by a 2 mm glass plate, and exposed to a hand-held UV light with the filter removed at a distance of 5 cm for 20 min. After irradiation, protein was solubilized in the dark by the addition of 0.1 ml of 0.05 M Tris-HCl (pH 7.5), 10 mM EDTA, 1% Triton X-100, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM phenylmethanesulfonyl fluoride at 0°C. Protein was precipitated with the addition of 100% (w/v) trichloroacetic acid to a final concentration of 10%. After 1 h at 0°C the proteins were precipitated by centrifugation and the pellet resuspended by brief sonication in 0.5 ml of 90% acetone/10% 0.1 N HCl at 0°C. After another hour at 0°C in the dark, protein was centrifuged down, the pellet air-dried overnight in the dark, and the proteins solubilized in 0.06 ml of SDS sample buffer [15] by heating to 95°C for 3 min. In the case of Triton X-114 phase equilibration experiment, 0.22 mg of rat and 0.43 mg of fat cell microsomes were photolabeled with 1.2 nmol ASA CoA in a final volume of 0.2 ml. After irradiation, the protein was diluted with 0.25 ml of 0.01 M Tris-HCl (pH 7.6)/0.15 M NaCl. 0.05 ml of 20% Triton X-114, phase equilibrated as described [16], was added and the suspension kept at 0°C in the dark for 5 min. After centrifugation at top speed on a microfuge for 5 min, the supernatent was removed from the detergent insoluble protein in the pellet, and layered over 0.8 ml of 0.25 M sucrose/0.06% Triton X-114/0.05 M Tris-HCl (pH 7.6)/0.15 M NaCl in a 1.5 ml centrifuge tube. After warming for 5 min at 32°C, the tube was centrifuged on a clinical centrifuge and the upper layer removed, made to 0.06% in Triton X-114, and the warming and centrifugation repeated. The upper layer (detergent-poor) and the combined lower layers (detergent-rich phase) were precipitated with trichloroacetic acid as above before separation of component proteins on SDS gel electrophoresis. Samples (typically 30 μl) were separated on 8–16% polyacrylamide gradient gels [15]. Broad range size marker proteins (Biorad) were included in each run. Gels were then fixed, stained with 0.1% Coomassie blue R-250, destained, dried, and exposed to Kodak XAR-5 film in the presence of an intensifier screen for different periods of time from 1 to 7 days.

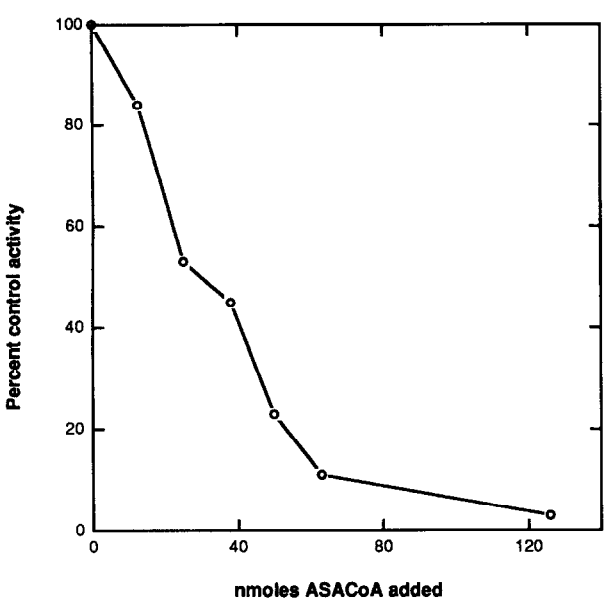

Fig. 1. Inhibition of DAGAT activity by ASACoA. DAGAT was assayed as described in section 2 using 5.5 μg of stripped liver microsomes, 10 μM [^{3}H]palmitoyl CoA (11 mCi/mmol), and the indicated amounts of ASACoA.

Fig. 2. Incorporation of [^{125}I]ASACoA into the neutral lipid fraction in the presence of dioleoylglycerol and rat liver microsomes. The DAGAT assay was carried out as described in section 2 using 11 μg of stripped rat liver microsomes (microsomes) or with the microsomes omitted (0). [^{125}I]ASACoA was added in place of [^{3}H]palmitate. The reaction was carried out at 25°C for 10 min and the reaction stopped by extraction of neutral lipids into the heptane phase. In each case the latter was dried under a stream of nitrogen, resuspended in heptane, and the neutral lipids chromatographed on a 0.25 mm Silica gel G plate in hexane/diethyl ether/glacial acetic acid (90:10:1). Appropriate standards were run in adjacent lanes and visualized by iodine vapors. Radiolabeled lipids were visualized by autoradiography. Triacylglycerol (TG) was identified by co-migration with authenic triolein. The spot marked X was not identified. The arrow marks the origin.

2.4. Other methods

DGAT activity was measured as described [13]. Protein concentrations were determined by the bicinchoninic acid assay with reagents supplied by Pierce [17].

3. Results

3.1. Inhibition of DGAT by ASACoA

The effect of ASACoA on the DGAT reaction was investigated to see if this fatty acyl CoA analogue could be recognized by the enzyme. ASACoA inhibited the DGAT reaction (Fig. 1). The reaction mix contained 25 μM [^{3}H]palmitoylCoA and was ~50% inhibited by 300 μM ASACoA. When

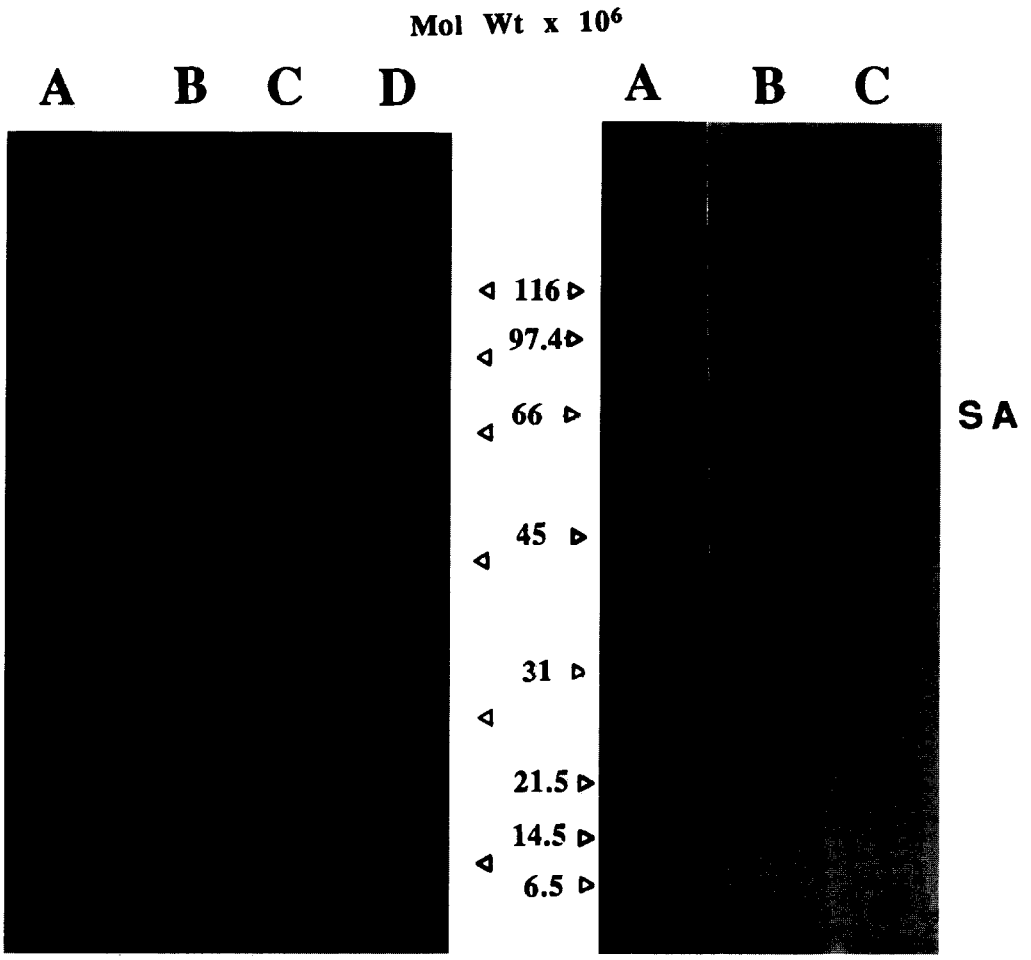

Fig. 3. SDS polyacrylamide gel separation of photolabeled rat liver (left) and epididymal fat cell (right) microsome preparations. Microsome preparations and photolabeling were as described in section 2. Lanes marked A are Coomassie blue stained lanes that are identical for corresponding autoradiographs in lanes B–D. Lanes B–D are autogradiographs of photolabeled proteins. Lanes B are from membranes photolabeled as described in section 2. Lanes C included 0.2 mM palmitoyl CoA (Sigma) prior to photolabeling. In lane D the photolabeling was carried out prior to addition of microsomes. The position of serum albumin (SA; 66 kDa) is indicated.

[^{125}I]ASACoA was added to the reaction in the absence of palmitoyl CoA, radiolabel was incorporated into two lipophilic compounds that could be extracted into the heptane phase and were separated by thin layer chromatography on silica gel G in hexane/diethyl ether/glacial acetic acid (90:10:1; Fig. 2, microsomes). The spot with the furthest migration (TG) co-migrated with authentic triolein while the other neutral lipid (X) was not identified. In this system phosphoglycerides, ASACoA, and dioleoylglycerol stayed at the origin. In the absence of enzyme (0), only a small amount of radiolabeled material at the origin was seen. These results show that ASACoA is recognized as a substrate by liver microsomal DGAT.

3.2. *Photolabeling of microsomes*

[^{125}I]ASACoA was then employed to photolabel microsomal membranes from two sources. As shown (Fig. 3) a band at ~66 kDa (SA) is labeled in both liver and epididymal fat cell microsome preparations. This band was shown by two-dimensional polyacrylamide gels to be serum albumin, carried over from blood in the case of rat liver, and from bovine serum albumin used in the collagenase digestion medium in the case of fat cells. Upon longer exposure a variable number of small bands of less than 25 kDa were also seen. None of the latter bands or that of serum albumin were greatly diminished in the presence of palmitoyl CoA. The remaining, major photolabeled bands were localized to 75, 54, 50 and 37 kDa in liver and 75, 64, 54 and 37 kDa in fat cell microsomes. As seen by examination of the corresponding Coomassie blue stained gel lanes for both liver and fat (A), photolabeled bands do not simply reflect the more abundant proteins in these membranes. All of the above photolabeled bands were diminished in the presence of palmitoyl CoA (lanes C) or myristyl CoA (not shown). No bands were seen when exposure to light preceded addition of microsomes in the case of rat liver (lane D) or fat cell (not shown) microsomes. Addition of co-substrates for acyltransferase reactions such as dioleoylglycerol (0.4 mM), 1-monooleoylglycerol (0.28 mM), 2-monooleoylglycerol (0.28 mM), or glycerol-3-phosphate (0.56 mM) to rat liver microsomes did not result in any noticeable change in the number or intensity of labeled bands.

3.3. *Fractionation of rat liver microsomes after photolabeling*

In order to enrich for photolabeled proteins, membranes were solubilized in 2% Triton X-114 and, after collecting insoluble proteins, solubilized proteins (in the supernatent after a 60 min centrifugation at 100,000 × *g*) were phase equilibrated into detergent-rich and detergent-poor fractions according to Bor-

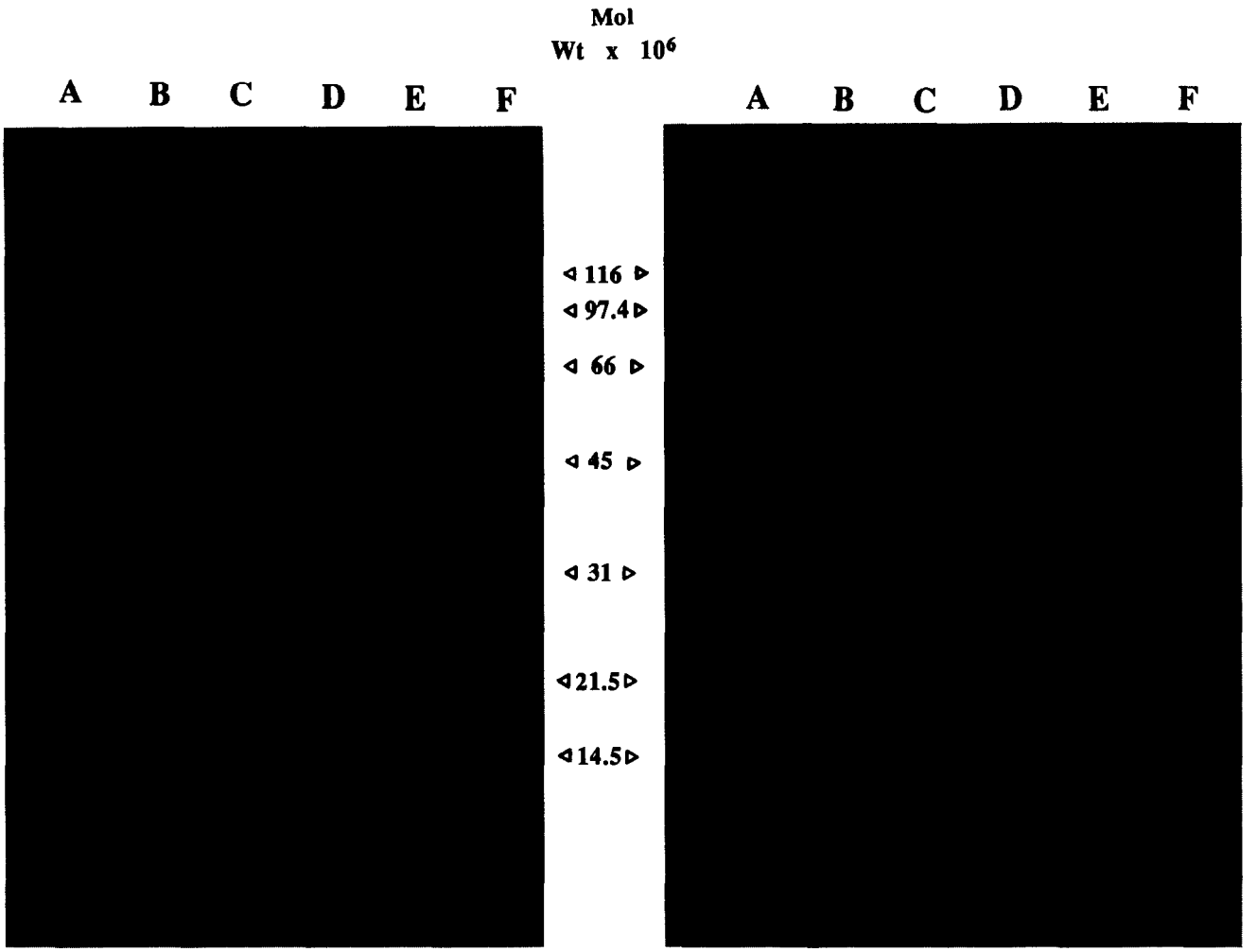

Fig. 4. SDS polyacrylamide gel separation of photolabeled rat liver and epidymal fat cell microsome preprations that have been solubilized in Triton X-114 and phase equilibrated. Protein samples were prepared as described in section 2, separated on polyacrylamide gels under denaturing conditions, stained with Coomassic blue, and then exposed to X-ray film. The photographed stained gel is on the left while the autoradiogram is on the right. Lanes A–C are from fat cells, lanes D–F are from liver. Lanes A and D: proteins insoluble in 2% Triton X-114; lanes B and E: proteins in the detergent-rich fraction; lanes C and F: proteins in the detergent-poor fraction. Palmitoyl CoA sensitive, photolabeled proteins are indicated with arrows.

lier [16]. All three fractions were precipitated with trichloroacetic acid and separated on 8–16% gradient polyacrylamide gels (Fig. 4). Photolabeled bands other than serum albumin were purified into the detergent-rich phase, indicating that all are integral membrane proteins that bind non-ionic detergent.

4. Discussion

A discrete number of integral microsomal proteins are shown to be photolabeled by [^{125}I]ASACoA in two cell types. The reduced labeling in the presence of palmitoyl CoA and the demonstrated interaction of ASACoA with one acyltransferase, DGAT, show that the photolabeling reagent is labeling proteins which have a limited number of fatty acyl CoA binding sites. By contrast, serum albumin, a contaminant in the microsome preparations, binds ASACoA in a manner insensitive to palmitoyl CoA.

Identical mobilities for three of the four photolabeled proteins from liver and fat may reflect similarities in enzyme content in these two tissues. Without further studies it is difficult to identify any of these labeled bands with a given enzyme. Fatty acyl CoA serves as a substrate for a variety of enzymes involved in lipid metabolism. Most of these participate in acylglycerol synthesis. Examples include *sn*-glycerol 3-phosphate acyltransferase, lysophosphatidic acid acyl transferase, dihydroxyacetone phosphate acyltransferase [18], as well as *sn*-1,2(2,3)-diacylglycerol acyltransferase [19]. These enzymes and others are localized to the microsome fraction of both liver and fat cell homogenates. One of the photolabeled proteins (75 kDa) is close to the reported monomer size (72 kDa) for DGAT determined from radiation inactivation experiments [4]. The identification of each of the photolabeled bands, however, with a specific acyltransferase activity must await the microsequencing of isolated, photolabeled proteins and the molecular cloning and expression of corresponding cDNAs in a null background. Previous attempts to isolate some of the acyl transferases have not been successful in identifying a single gel band with activity because of the lability of activity upon solubiliza-

tion and purification. The identification of a limited number of proteins described here represents a good starting point for the purification of proteins without relying on enzyme activity.

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