[88] 1,2-Diacyl-*sn*-glycerol: Sterol Acyl Transferase from Spinach Leaves (*Spinacia olerecea* L.)

By R. E. GARCIA and J. B. MUDD

The synthesis of sterol esters in animals has been reported to follow three mechanisms: (*a*) phosphatidylcholine: cholesterol acyltransferase[1]; (*b*) fatty acyl-CoA: cholesterol acyltransferase[2]; and (*c*) reversal of cholesterol esterase.[3] In the plant kingdom Bartlett *et al.*[4] reported sterol ester biosynthesis in *Phycomyces blakesleeanus* to be catalyzed by a phosphatidylcholine: sterol acyltransferase. In yeast the synthesis appears to be by the acyl-CoA pathway.[5] In our studies,[6–10] we found that [^{14}C]acyl-labeled phosphatidylcholine gave rise to labeled sterol esters, but product analysis showed that sterol ester biosynthesis continued after phosphatidylcholine was depleted in the reaction mixture. A major labeled compound in this study was diacylglycerol, and we tested this as an acyl donor. It was by far the most efficient acyl donor of those tested.

$$\text{Sterol} + \text{diacylglycerol} \rightarrow \text{sterol ester} + \text{monoacylglycerol}$$

Assay Method

Principles

The enzyme preparation is added to a mixture of sterol and 1,2 diacyl-*sn*-glycerol in a micellar system that includes Triton X-100 and phosphatidylcholine in addition to the substrates. Either the sterol or the acyl moieties of the 1,2 diacyl-*sn*-glycerol is radioactive. After the incubation period, the reaction mixture is partitioned into aqueous and chloroform phases. An aliquot of the chloroform phase is subjected to thin-layer chromatography (TLC). The sterol ester product is located by iodine

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spray or by autoradiography. The sterol ester area is scraped off the plate, and the radioactivity is determined by scintillation counting either with or without elution of the sterol ester from silica gel.

Reagents

Radioactive sterols such as [4-^{14}C]sitosterol can be obtained from Amersham/Searle Corporation, Arlington Heights, Illinois; [^{14}C] Diacylglycerols can be obtained from Dhom Products, Los Angeles, California. Nonradioactive sterols can be purchased from Applied Science Laboratories, State College, Pennsylvania. Nonradioactive diacylglycerols were obtained from Nu-Chek-Prep, Elysian, Minnesota. All other chemicals were obtained from Sigma Chemical Company, St. Louis, Missouri.

Procedure

Preparation of Mixed Micelles. Micelles containing Triton X-100, phosphatidylcholine, diacylglycerol, and cholesterol were prepared by adding 1.2 mg of phosphatidylcholine, 1.2 mg of diacylglycerol, 1.2 mg of sterol, and 12 mg of Triton X-100 to a test tube. The organic solvents in which the compounds were dissolved were removed by evaporation under reduced pressure. Either the sterol or the diacylglycerol was radioactive. Distilled water was added dropwise to the detergent–lipid mixture with frequent mixing until the volume was 4 ml. The resulting mixed micelles appeared like a perfectly clear solution and remained so when stored at 5°.

Reaction Mixtures. The standard assay mixture contained 70 m*M* MES-NaOH, pH 7, 75 μM cholesterol (10^5 cpm), 52 μM diacylglycerol, 40 μM phosphatidylcholine, 480 μM Triton X-100, 10 mg of bovine serum albumin, and 2.5 mg of the enzyme preparation in a final volume of 1 ml. The concentrations of detergent and lipids correspond to 0.1 ml of the mixed micelle solution added to each 1 ml of reaction mixture. Reactions were started by addition of the enzyme and run in a Dubnoff metabolic shaker at 30° for 10 min. The reaction was stopped by addition of 3 ml of chloroform : methanol (1 : 2, v/v). Isolation of the lipid fraction followed the method of Bligh and Dyer.[11]

Separation of Products. (a) Separation of sterol esters from other lipids: The lipid sample in chloroform was applied to a thin-layer plate coated with silica gel G. The plates were developed in one dimension in benzene : chloroform (40 : 60, v/v). Sterol ester (R_f 0.75) was well separated

[11] E. G. Bligh and W. J. Dyer, *Can. J. Biochem. Physiol.* **37**, 911 (1959).

from sterol (R_f 0.1). The sterol ester spot was scraped off for assay by scintillation counting. (*b*) Separation of molecular species of sterol ester: Cholesterol esters with fatty acid substituents of varying degrees of saturation were separated by TLC on silica gel G plates impregnated with 2% (w/w) $AgNO_3$. The plates were developed in benzene : hexane (1 : 1, v/v) as described by Goodman *et al.*[2] Radioactive areas were detected by autoradiography and scraped off the plates, and the sterol esters were eluted with chloroform. The eluted lipid was assayed in a scintillation counter.

Preparation of Enzyme

Differential centrifugation of the leaf homogenate (specific activity, 0.2 nmol/mg protein per minute) showed the 20,000 *g* pellet to have the highest amount of enzyme of the fractions tested (34% of enzyme units, 1.4 nmol/mg protein per minute), but the highest specific activity was in the 88,000 *g* pellet (23% of enzyme units, 2.2 nmol/mg protein per minute). These two pellets were examined by electron microscopy and were devoid of chloroplasts, mitochondria, and fragments of these organelles. Endoplasmic reticulum, ribosomes, and other membrane vesicles were present in the active fractions. No further attempt has been made to determine the subcellar location of the enzyme.

Acetone Powder. Petioles were removed from leaves of spinach (*Spinacia oleracea* L.), the leaves were washed in distilled water and chilled at 5°. The leaves (200 g) were minced in a food chopper and then homogenized in a Waring blender in 300 ml of a solution containing 450 m*M* sucrose, 10 m*M* Tris-HCl, pH 7.5, and 1 m*M* EDTA at 0° with four bursts of 2 sec duration. The homogenate was filtered through three layers of cheesecloth. The fraction sedimenting between 3000 and 20,000 *g* was collected and suspended in 4 ml of 100 m*M* Tris-HCl, pH 7.5. The suspension was added slowly to 70 ml of rapidly stirred acetone at −15° or 0°. Acetone at 0° tends to give better extraction of lipids. The precipitated protein was sedimented by centrifugation, washed by resuspension in cold acetone, and sedimented again by centrifugation. The pellet was then dried under reduced pressure at room temperature. This procedure gave approximately 400 mg of acetone powder from 200 g of leaves. The dried powder was stored at −15°. Preparation of the acetone powder is useful because it removes lipids that could participate in the enzyme-catalyzed reaction and because the preparation can be stored; it does not improve the specific activity of the enzyme.

Acetone–Ether Powder. The procedure described above was modified in that the pellet obtained in the first acetone precipitation was suspended

in diethyl ether at 0° rather than in acetone. The suspension was then centrifuged, and the resulting pellet was dried under reduced pressure.

The acetone or acetone–ether powders were resuspended in buffer for addition to reaction mixtures: 50 mg of acetone or acetone–ether powder were suspended in 2 ml of 100 m*M* MES–NaOH buffer and mixed with a Potter–Elvehjem glass homogenizer.

Properties

Stability. The acetone and acetone–ether powders are stable for several weeks when stored in a desiccator at −15°.

Substrate Specificity. Cholesterol, sitosterol, and campesterol do not seem to be distinguished by the enzyme. Cholesterol and sitosterol were compared at several time points and several concentrations. All three sterols were tested in competition studies and appeared to be equivalent. These results are reasonably consistent with analyses of sterol esters isolated from plant tissue.[12]

The testing of diacylglycerols with only one type of fatty acid indicated the following preference for fatty acid transfer 16:1 > 18:3 > 18:1 > 18:2 > 16:0 > 18:0 ≥ control. This order was found both in time course and diacylglycerol concentration studies. In all cases it could be readily demonstrated by $AgNO_3$ TLC of the sterol esters, that the fatty acid of the supplied diacylglycerol was transferred to the sterol. However, when mixtures of diacylglycerol were used in mixed micelles, e.g., 13 μM each of 16:0, 18:1, 18:2, and 18:3 diacylglycerol, the preference was 18:3 > 16:0 > 18:2 > 18:1, the most striking feature being the greatly increased efficacy of 16:0 diacylglycerol when present in the mixture of diacylglycerols.[9] It is not known whether there is preferential transfer of an acyl group from a particular position of the diacylglycerol. Neither mono- nor triacylglycerol is a suitable substrate for sterol ester biosynthesis.

Other Substrates. The enzyme extract shows several acyltransferase activities. Both di- and monoacylglycerol are hydrolyzed in the presence of the enzyme (transfer to water). In the presence of ethanol, ethyl esters of fatty acids are formed, particularly from monoacylglycerol (transfer to ethanol). In the presence of diacylglycerol, some triacylglycerol is formed, indicating acyl transfer from one diacylglycerol to the vacant hydroxyl of another.

Activators and Inhibitors. Tween 80 was not as satisfactory as Triton X-100 for the preparation of micelles. Phosphatidylcholine was indispens-

[12] J. V. Torres and F. Garcia-Olmedo, *Biochim. Biophys. Acta* **409**, 367 (1975).

able in the micelles for activity to be observed. Digitonin was an effective inhibitor of the reaction.

In earlier studies of sterol ester biosynthesis by enzyme preparations from spinach leaves, a pH optimum of 6.0 in MES–NaOH buffer was determined.[7] But diacylglycerol was not added to the reaction mixtures; it was generated from phospholipids by the action of phospholipase D and phosphatidic acid phosphatase. In fact it is difficult to measure a strong dependence on added DG at pH 6. Two methods can be employed to show this dependence. The simplest is to raise the pH to 7 to avoid the action of phospholipase D and phosphatidic acid phosphatase. The second is the judicious use of metal ions that will inhibit the phospholipid metabolizing enzymes while having a minimal effect on the 1,2-diacylglycerol : sterol acyltransferase. For this purpose Zn^{2+}, Cu^{2+}, and Fe^{2+} are particularly effective at 1 m*M* concentration.

[89] Aldehyde Dehydrogenases from Liver

EC 1.2.1.3 Aldehyde : NAD^+ oxidoreductase

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Aldehyde dehydrogenase (EC 1.2.1.3) catalyzes irreversible dehydrogenation of a large variety of aldehydes to corresponding carboxylic acids utilizing NAD as coenzyme. The enzyme occurs in a variety of organs, but its concentration is highest in the liver.[1,2] It was first demonstrated in beef liver by Racker (1949),[3] followed 20 years later by purification to homogeneity of a single enzyme from horse liver.[4] Subsequent purifications, however, have conclusively demonstrated that aldehyde dehydrogenase occurs in multiple molecular forms (isozymes) that differ in primary structure, catalytic properties, electrophoretic mobility, and subcellular distribution.[5–8] Two aldehyde dehydrogenases have been purified to homogeneity from horse[5] and sheep[6] liver and demonstrated to belong to

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