MEMBRANES AS ACCEPTORS FOR PALMITOYL CoA IN FATTY ACID BIOSYNTHESIS

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1. Introduction

The biosynthesis of phospholipids involves an interesting interplay between ble and membrane-bound enzymes. The final assembly of phospholipid molecules is catalyzed by membrane-bound enzymes [1]; however the precursor substrates, palmitoyl CoA and stearoyl CoA*, are synthesized (in yeast) by the soluble multienzyme complex fatty acid synthetase (FAS). As is well known palmitoyl CoA associates easily and unspecifically with a variety of soluble enzymes and causes their denaturation [2, 3]. In line with this, the present study shows that newly synthesized palmitoyl CoA cannot freely dissociate from the FAS. Therefore a free diffusion of individual palmitoyl CoA molecules is highly improbable.

Thus the question arises how is palmitoyl CoA transferred from the FAS to the subsequent membrane-bound enzymes. The rate and mechanism of this transfer may be important for the regulation of the lipid biosynthesis. The simplest mechanism might be the transfer through direct collisions between the FAS and the membrane-bound enzymes. A slightly more sophisticated one might involve lipid binding molecules, like bovine serum albumin (BSA), operating as carriers of palmitoyl CoA. In both cases the transfer would be accomplished through the three-dimensional diffusion of macromolecules.

Alternatively, it is conceivable that a combined mechanism operates in which palmitoyl CoA is introduced into target membranes and diffuses in the plane of the membrane (cf. Adam and Delbruck [4]). This model presupposes that i) lipid membranes can serve as acceptors (and as a reservoir) for palmitoyl CoA and ii) that the palmitoyl CoA molecules can rapidly diffuse within the plane of the membrane. The present paper provides experimental evidence in favour of this mechanism.

2. Materials and methods

The assay and purification of the fatty acid synthetase from yeast followed procedures described in [5]. Acetyl CoA, malonyl CoA and palmitoyl CoA were prepared according to [6–9].

BSA, CoASH and NADPH were commercial products from Behringwerke, Marburg and Boehringer, Mannheim, respectively. [^{14}C]Acetic anhydride was obtained from the Radiochemical Centre, Amersham, and 1-anilino-napththalene-8-sulfonate (ANS^-) from Pierce Chemicals.

E. coli K 1062 plasma membranes were kindly supplied by Dr. P. Overath, Köln. They were prepared according to Kaback's method [10].

Dimyristoyllecithin was a product from Koch–Light. The lipids were ultrasonically dispersed at 30° for 3–5 min to yield optically clear solutions.

The kinetics of palmitoyl CoA synthesis was measured by following NADPH consumption using a

* The term "palmitoyl CoA" will be used below to denote both compounds.

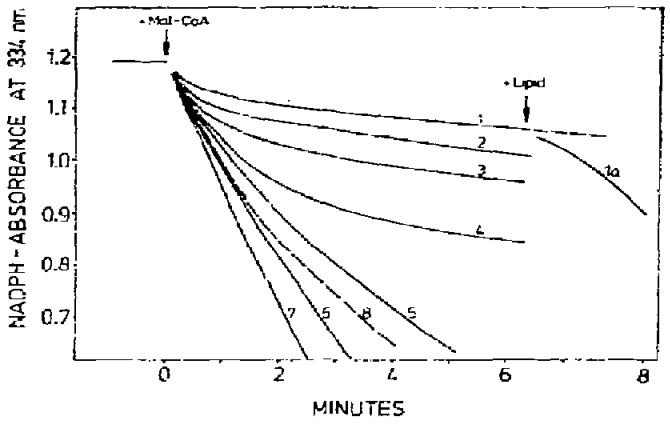

Fig. 1. Effect of lipid acceptor substances on the kinetics of fatty acid synthesis (as measured by the NADPH consumption). The incubation mixture (2 ml) contained: 200 μmoles potassium phosphate pH 7.5; 0.4 μmole NADPH; 0.2 μmoles acetyl CoA and 20 μg fatty acid synthetase (2000 mU/mg). The reaction was initiated by the addition of 0.2 μmole malonyl CoA. Curve (1): Incubation mixture without acceptor substances; (1a): After a prolonged period of self-inhibition dimyristoyllecithin bilayers were added (3.8×10^{-5} M lipid). Curves (2)–(6): increasing concentrations of dimyristoyllecithin dispersions: (2) 3.1×10^{-6} M; (3) 6.2×10^{-6} M; (4) 12.5×10^{-6} M; (5) 5×10^{-5} M; (6) 1.1×10^{-4} M. Curve (7): In the presence of 1 mg bovine serum albumin (about 7.5×10^{-6} M). Curve (8): In the presence of *E. coli* plasma membranes in a concentration corresponding to 2.5×10^{-5} M lipid.

Cary 14. A total of 14 NADPH molecules is consumed for 1 palmitoyl CoA. The fluorescence data were obtained with a Fica 55, an instrument allowing the recording of fully corrected differential spectra.

3. Results

Palmitoyl CoA and stearoyl CoA, the products of the FAS, are effective inhibiters of the enzymatic activity of FAS [11]. This is demonstrated by curves 1 and 7 in fig. 1. These curves show the kinetics of the fatty acid synthesis in the presence (curve 7) and in the absence (curve 1) of BSA which is a powerful acceptor of fatty acid derivatives. In the absence of BSA the enzymatic activity is blocked almost completely after a short period of synthesis (rest activity about 5%); this initial period of synthesis will be denoted in the following as the "active period". In the

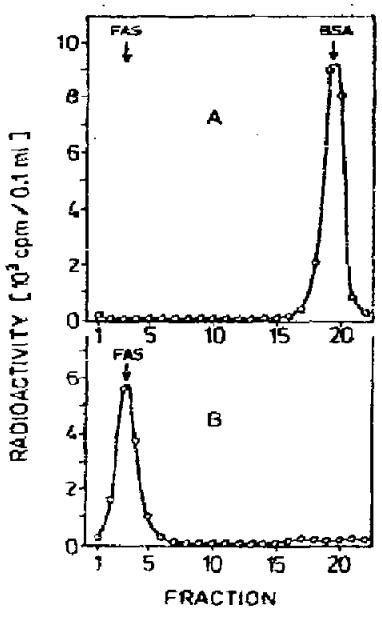

Fig. 2. Assay for the association of the products of fatty acid synthesis (long-chain [^{14}C]acyl CoA) with the fatty acid synthetase (FAS) and bovine serum albumin (BSA) by sucrose density gradient centrifugation. The incubation mixture (2 ml) contained: 200 μmoles potassium phosphate pH 7.5; 0.4 μmole NADPH; 0.018 μmole [^{14}C]acetyl CoA (10 μCi/μmole) and 100 μg fatty acid synthetase (2000 mU/mg). The reaction was initiated by the addition of 0.2 μmole malonyl CoA. Incubation: 5 min at 25°. (A) Incubation in the presence of 2 mg BSA. (B) Without BSA (product-inhibition occurs soon). Aliquots of the incubation mixtures were layered on a linear sucrose density gradient (5–20%) in 0.1 M potassium phosphate pH 6.5. After centrifugation for 5.5 hr at 40 000 rpm and 10° (SW 40, Beckman L 2-65) the fractions were analysed for long chain [^{14}C]acyl CoA in the following way: 0.2 mg BSA and 2 ml 5% trichloroacetic acid were added to each fraction; the precipitates were collected on millipore filters (HAWP 0.45 μm) washed 6 times with 5% trichloroacetic acid and assayed for radioactivity after drying

presence of BSA the effect of product-inhibition is not observed. It can be shown that the palmitoyl CoA molecules are readily transferred from the FAS to BSA and that they remain quantitatively attached to the FAS in the absence of BSA. Using [^{14}C]acetyl CoA as substrate the synthesis was carried out (A) in the presence and (B) in the absence of BSA and the association and spatial distribution of [^{14}C]palmitoyl CoA was analyzed by sucrose density gradient centrifugation. The results of this analysis are plotted in fig. 2. In case (A) the [^{14}C]palmitoyl

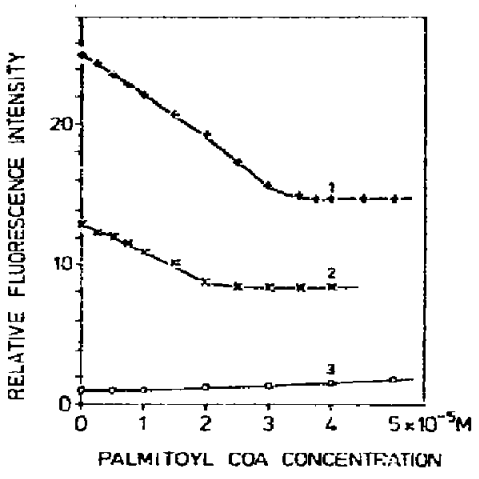

Fig. 3. Decrease in ANS^- fluorescence intensity (340 nm, 500 nm) upon addition of palmitoyl CoA to dimyristoyllecithin dispersions; 25°. ANS^- conc.: 1.25×10^{-4} M; 0.1 M potassium phosphate pH 7.5. Curve (1): 1.25×10^{-4} M lecithin; Curve (2): 0.62×10^{-4} M lecithin; Curve (3): Interaction of ANS^- with palmitoyl CoA.

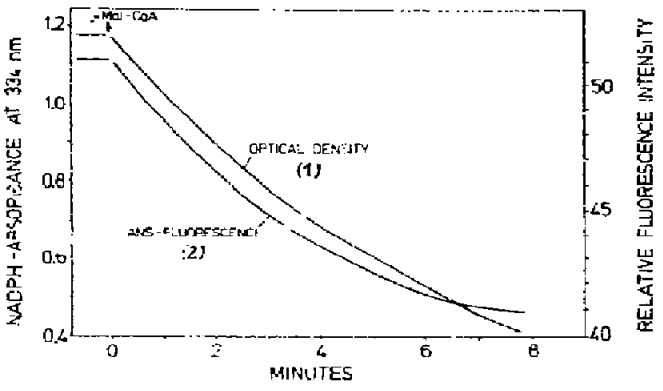

Fig. 4. Comparison of the kinetics of palmitoyl CoA synthesis (1) and of the incorporation of palmitoyl CoA into dimyristoyllecithin bilayers (2). Curve (2) shows the decrease in ANS^- fluorescence intensity (410 nm, 520 nm) in the course of the reaction (cf. fig. 3). Identical incubation mixtures were used for both experiments containing 0.62×10^{-4} M lipid and 5×10^{-5} M ANS^- in addition to the substrates used for experiment 1 in fig. 1. The fluorescence decrease was measured against an identical incubation mixture containing no ANS^- (differential spectrum).

CoA sediments quantitatively with BSA whereas in case (B) it remains associated with the FAS. Palmitoyl CoA was not found in detectable amounts in free solution. This suggests that direct collisions between the FAS and BSA are necessary to transfer the palmitoyl CoA molecules from the FAS to BSA. From curve 1 of fig. 1 one estimates that about 125–150 palmitoyl CoA molecules are synthesized per FAS complex (M.W. = 2.3×10^6) in the "active period". Thus the net production of palmitoyl CoA in a given incubation mixture during the "active period" is expected to increase linearly with the enzyme concentration. This is fully supported by the relevant experiments (not shown).

Curves 2 to 6 in fig. 1 demonstrate that natural membranes and lipid bilayers are also powerful acceptors of long-chain acyl CoA compounds from the FAS. These curves show the kinetics of the fatty acid synthesis in the presence of increasing amounts of dimyristoyllecithin bilayers. Curve 8 shows the result of analogous experiments in which *E. coli* plasma membranes served as acceptors. The net amount of palmitoyl CoA that can be synthesized in the "active period" increases linearly with the lipid concentration, indicating that a given lipid matrix has a well-defined capacity for the adsorption of palmitoyl CoA. From fig. 1 one estimates that as much as 17–20 palmitoyl CoA molecules can be incorporated per 100 lecithin molecules.

Fig. 1 shows that the activity of the product-inhibited enzyme can be restored by the addition of phospholipid vesicles (curve 1a in fig. 1).

Two experiments were performed to check whether palmitoyl CoA is actually incorporated into the lipid layers. A substitution titration was carried out using the fluorescence probe ANS^- as a substituent and indicator. Second, the effect of palmitoyl CoA on the thermal phase transition of dimyristoyllecithin was studied using optical measurements [13, 14].

i) Adsorption of ANS^- to lipid membranes results in a strong enhancement of the fluorescence intensity (cf. review [12]). When palmitoyl CoA is added to a lipid dispersion containing ANS^-, the fluorescence intensity decreases, indicating the release of ANS^- from the membrane surface. One obvious reason for this effect is the electrostatic repulsion of the ANS^- by the incorporated palmitoyl CoA which carries 3–4 negative charges per molecule. As shown in fig. 3 the fluorescence intensity reaches a plateau for palmitoyl CoA concentrations larger than a value c^*, characteristic for the lipid concentration. From the values of

c^* determined by the curves in fig. 3 we estimate that a maximum number of 25 palmitoyl CoA molecules can be incorporated into the lipid layers per 100 lipid molecules. The fluorescence decrease accompanying the incorporation of palmitoyl CoA can be utilized to measure independently the rates of synthesis (NADPH-consumption) and incorporation of palmitoyl CoA. As demonstrated in fig. 4 these two processes are synchronized.

ii) Addition of palmitoyl CoA to dispersions of dimyristoyllecithin causes a significant broadening and a gradual disappearance of the lipid phase transition at 24°. This effect is characteristic of the incorporation of foreign molecules into a pure lipid matrix [15].

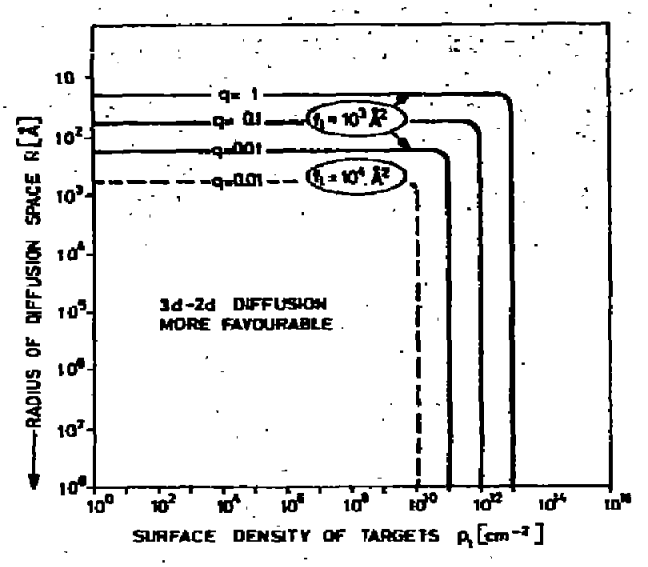

Fig. 5. Contour lines enclosing the regime in which the combined 3d–2d diffusion mechanism is more favourable than free diffusion alone (calculated according to eq. (4), Appendix). q denotes the ratio $\tau_{(3/2)}/\tau_{(2)}$ of the respective transfer times; f_t is the target area. Similar contour lines are obtained if R is considered as constant and increasing values of $D_{(3)}/D_{(2)}$ are drawn on the ordinate scale.

4. Discussion

The products of the fatty acid synthetase, palmitoyl CoA and stearoyl CoA, remain attached to the multienzyme complex and inhibit the enzymatic activity unless lipid accepting molecules, like BSA, or lipid membranes are present in the solution. Bilayers of dimyristoyllecithin can incorporate as much as 20 palmitoyl CoA molecules per 100 lipid molecules. The acceptor *capacity* of a given membrane is most probably determined by fixed membrane charges and by the negative charges of the incorporated palmitoyl CoA.

On the basis of our findings we propose the following mechanism for the transfer of palmitoyl CoA from the FAS to subsequent membrane-bound enzymes. In a first step the palmitoyl CoA is carried to a place somewhere on the membrane by its synthesizing enzyme or, perhaps, by another lipid-carrier molecule. (At present there is no evidence that the FAS is associated with membrane surfaces [18]). Lateral diffusion within the plane of the membrane would be the mechanism by which the palmitoyl CoA is transferred to the subsequent membrane-bound enzymes. The following findings lend support to this hypothesis: i) In a recent study Adam and Delbruck [4] have shown that a transfer mechanism, involving lateral diffusion in addition to free diffusion permits a much faster transfer of a molecule from the cell cytoplasma to a small target on the cell membrane than free diffusion alone. A detailed discussion of this point is given in the Appendix. (ii) The occurence of rapid lateral diffusion of lipid soluble molecules in lipid bilayers and natural membranes has been established in recent ESR studies [16, 17]. The coefficients of lateral diffusion for androstane and fatty acids are as high as $(1-3) \times 10^{-8}$ cm^2/sec, corresponding to a net displacement of these molecules by about 10 000 Å in one second. Therefore palmitoyl CoA molecules are also expected to be highly mobile within the plane of a lipid matrix. In addition to a higher transfer rate the combined mechanism would also avoid complications due to the unspecific association of palmitoyl CoA with soluble proteins within the cell (detergent properties of palmitoyl CoA).

According to this model a natural membrane containing palmitoyl CoA can be visualized as a resevoir of substrates for the enzymes involved in the further assembly of lipid molecules (glycerolphosphate acyltransferases, desaturases, etc.). The rate of fatty acid biosynthesis would then be governed by the acceptor capacity and the degree of saturation of the membrane reservoir – in close analogy to the investigated model systems. This interplay may be important for the regulation of the lipid biosynthesis.

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Appendix: ***The advantage of lateral diffusion in the transfer of palmitoyl CoA from the fatty acid synthetase to membrane bound enzymes.***

We consider a spherical diffusion cell (radius R) containing randomly diffusing FAS molecules which are ideally reflected by the surrounding membrane. Thus, macroscopic concentration gradients do not exist and the average time $\tau'_{(3)}$ required by an FAS molecule to hit the membrane is governed by the self-diffusion of the FAS. (The index (3) is used for three-dimensional diffusion). $\tau'_{(3)}$ is given by

$$\tau'_{(3)} = c R^2/D_{(3)} \qquad (1)$$

where $D_{(3)}$ denotes the coefficient of free (three-dimensional) diffusion ($D_{(3)}$ (H_2O, 25°) $\approx 2 \times 10^{-7}$ cm^2/sec for the FAS). Lower and upper limits for c are 1/20 and 3/2. These values are obtained by averaging over the shortest and longest times of travel from all places in the cell to the enclosing membrane. In the following a mean value of $c = 3/4$ will be used.

We are interested in the average time (τ) required for the transfer of palmitoyl CoA from the FAS to target molecules on the membrane for the following two cases: i) the transfer is accomplished through direct collisions between the freely diffusing FAS molecules and the membrane bound targets ($\tau_{(3)}$); ii) the palmitoyl CoA is incorporated randomly into the membrane (via collisions between the FAS and the membrane) and reaches the target molecules through lateral diffusion within the plane of the membrane ($\tau_{(3/2)}$). It will be assumed: (1) that the area of all targets is negligibly small compared to the rest of the membrane, (2) that the targets represent "sinks" of infinite capacity and (3) that following the release of palmitoyl CoA from the FAS new palmitoyl CoA molecules are synthesized in a time which is short compared to the transfer times (τ). The average time $\tau_{(3)}$ required by a freely diffusing FAS molecule to hit a target on the membrane is

$$\tau_{(3)} = \tau'_{(3)}/\rho_t f_t \qquad (2)$$

where ρ_t denotes the density of target molecules per cm^2 of the membrane surface and f_t is the surface area of one target molecule.

For the combined mechanism (free and lateral diffusion) the average time of transfer is

$$\tau_{(3/2)} = \tau'_{(3)} + \tau_{(2)}$$

where $\tau_{(2)}$ denotes the average time of lateral diffusion from an arbitrary place on the membrane to a target molecule. Since the target molecules represent sinks for the palmitoyl CoA, concentration gradients will be established within the plane of the membrane. For an exact treatment of the diffusion problem within the membrane one has to start from Fick's second law of diffusion and assumptions must be made about the distribution and density of sinks and sources. However, an approximate value for $\tau_{(2)}$ can be estimated easily if we consider the lateral diffusion of the palmitoyl CoA as a random walk problem (self-diffusion) involving a series of successive jumps of length λ within the two-dimensional lattice of lipid molecules.

One derives (cf. [16])

$$\tau_{(2)} = \frac{1}{2(r_d + r_t)\, l \rho_t} = \frac{\lambda}{4(r_d + r_t)\rho_t D_{(2)}} \qquad (3)$$

where l denotes the integrated diffusional path in 1 sec ($l = D_{(2)}/2\lambda$) and $(r_d + r_t)$ is the sum of the radii of the diffusing molecule (palmitoyl CoA) and the target molecule. Using the values $D_{(3)} = 2 \times 10^{-7}$ cm^2/sec, $D_{(2)} = 2 \times 10^{-8}$ cm^2/sec, $\lambda = 8$ Å (cf. [16]) and assuming $(r_d + r_t) \approx 50$ Å we obtain for the ratio q of the transfer times:

$$q = \frac{\tau_{(3/2)}}{\tau_{(3)}} = f_t \rho_t \left(1 + \frac{\lambda}{3\rho_t (r_d + r_t)} \frac{1}{R^2} \frac{D_{(3)}}{D_{(2)}}\right) \approx f_t \left(\rho_t + \frac{1}{2R^2}\right) \qquad (4)$$

For a given ratio of the diffusion coefficients ($D_{(3)}/D_{(2)} \approx 10$ in our case) the ratio of the transfer times is determined by the area (f_t) and density (ρ_t) of the target molecules and by the radius, R, of the diffusion space.

The combined transfer mechanism becomes more favorable (q decreases) with decreasing density of targets and increasing size of the diffusion space. In fig. 5 contour lines are drawn in an R versus ρ_t diagram enclosing the regimes in which the combined mechanism is more favorable ($q < 1$). For a given area of the target molecules (f_t) the combined mechanism is more favorable if i) the radius of the diffusion space is larger than a critical value R^{cr} and if ii) the density of target molecules is lower than a critical value ρ_t^{cr}.

Defining the "more favorable" regime by $q \leq 0.1$ we obtain $R^{cr} \approx 200$ Å, $\rho_t^{cr} = 10^{11}/\text{cm}^2$ for $f_t = 10^4$ Å^2; and $R^{cr} = 80$ Å, $\rho_t^{cr} = 10^{12}/\text{cm}^2$ for $f_t = 10^3$ Å^2. This shows that in the case considered ($D_{(3)}/D_{(2)} \approx 10$) an extremely wide range of parameters (f_t, ρ_t, R) exists for which the combined mechanism (free and lateral diffusion) is more favourable than free diffusion alone.

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