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COENZYME A: FATTY ACID SYNTHETASE APOENZYME 4'-PHOSPHOPANTETHEINE TRANSFERASE IN YEAST

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SUMMARY: A mixture of two pantetheine-free mutant fatty acid synthetases was dissociated and recombined in vitro to form a hybrid apoenzyme complex. In vivo the corresponding Saccharomyces cerevisiae fas-mutants exhibit interallelic complementation when crossed with each other and the enzyme synthesized in the resulting diploid contains pantetheine and exhibits overall fatty acid synthetase activity. Accordingly, the hybrid apoenzyme formed in vitro could be activated to holo-fatty acid synthetase when incubated with coenzyme A and a partially purified yeast cell extract. The enzyme coenzyme A: fatty acid synthetase apoenzyme 4'-phosphopantetheine transferase has thus been identified in yeast. Further studies on the mechanism of fatty acid synthetase holoenzyme formation will now be possible.

INTRODUCTION

All fatty acid synthetases so far studied contain enzyme-bound phosphopantetheine as a prosthetic group (1). As shown by Schweizer et al. (2) the yeast fatty acid synthetase complex accomodates 5-6 phosphopantetheine residues per enzyme molecule. This number is in good agreement with the $\alpha_6\beta_6$ molecular structure proposed for yeast fatty acid synthetase (3). In this hexamer phosphopantetheine is, together with the ß-ketoacyl synthetase and ß-ketoacyl reductase component enzymes, a part of the multifunctional subunit α (3).

As indicated by studies of Elovson and Vagelos (4), phosphopantetheine is attached to the acyl carrier protein (ACP) of the E.coli fatty acid synthetase system by an enzyme catalyzing the reaction:

$$\text{CoA + apo-ACP} \xrightarrow{Mg^{2+}} \text{holo-ACP + 3',5'-adenosinge diphospate}$$

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By the same mechanism phosphopantetheine seems to be ligated also to animal fatty acid synthetase complexes (5). In yeast, a corresponding reaction has not been identified, so far. This has been mainly due to the lack of a suitable fatty acid synthetase apoprotein. Although pantetheine-free mutant fatty acid synthetases from yeast are known, their phenotype has been ascribed to a defective apoenzyme rather than to an inactivated ligase (6).

Only recently, the technique of reversible dissociation of yeast fatty acid synthetase as described by Wieland and Lynen (7) allowed the construction of appropriate fatty acid synthetase apoproteins capable of being converted into the corresponding holo-enzymes. Certain pairs of pantetheine-free fatty acid synthetase mutants, when crossed with each other, are known to form diploids synthesizing an active holoenzyme (8). As reported in this communication, this *in vivo* complementation can be reproduced *in vitro* by dissociation and subsequent reassociation of a mixture of the two apoenzymes isolated from the corresponding mutants. Thereby, a hybrid apoenzyme is formed which can be activated, in the presence of coenzyme A, to fatty acid synthetase holoenzyme by a fatty acid synthetase-independent enzyme present in the crude yeast cell homogenate.

MATERIALS AND METHODS

Yeast strains: The *Saccharomyces cerevisiae* fatty acid synthetase (*fas*) mutants used in this study had been isolated previously in this laboratory. Details of the isolation procedure as well as the general genetic and biochemical characteristics of *fas*-mutants have been described in earlier publications (3,9).

Enzyme isolation and assays: Wild type and mutant fatty acid synthetase complexes were purified from cells harvested from 70 liter fermenter cultures (Chemap). The purification procedure was essentially as described by Schweizer et al. (6). Overall fatty acid synthetase activity as well as the ß-ketoacyl reductase and palmityl transferase component enzyme activities were assayed according to Lynen (10). The activities of the acetyl- and malonyl-transferase, the enoyl reductase, the dehydratase and the ß-ketoacyl synthetase component enzymes were assayed as indicated by Weithmann (11). Radioactive labeling of enzyme-bound pantetheine was achieved by growing yeast cells in the presence of 1-[^{14}C]-pantothenic acid which was chemically synthesized from 1-[^{14}C]-ß-alanine (NEN-chemicals, spec.act. 50 mCi/mmole)according to Wieland et al. (12).

In vitro recombination of mutant fatty acid synthetases: For dissociation and subsequent reassociation of purified yeast fatty acid synthetases the procedure indicated by Wieland and Lynen (7) was used with slight modification (manuscript in preparation).

RESULTS AND DISCUSSION

In vivo and in vitro complementation between pantetheine-free fatty acid synthetase mutants. As already reported previously (8) the two pantetheine-deficient fatty acid synthetase mutants fas 2-750 and fas 2-655 exhibit intragenic complementation when crossed with each other. Phenotypically, this complementation may be recognized by the ability of the resulting diploid to grow in the absence of dietary fatty acids (Fig. 1). In accordance with these growth characteristics the fatty acid synthetase complex isolated from fas 2-750/fas 2-655 diploid cells exhibits overall fatty acid synthetase and ß-ketoacyl synthetase activities although both of the activities were lacking in each of the two parent strains (Table 1). As it is frequently observed with intragenic complementation (13) the specific activity of the hybrid enzyme synthesized in the diploid cell does not attain full wild type levels. Thus, the reactivation of overall fatty acid synthetase and ß-ketoacyl reductase activities observed in the hybrid enzyme may be considered as a consequence of the restored pantetheine binding capacity of this enzyme.

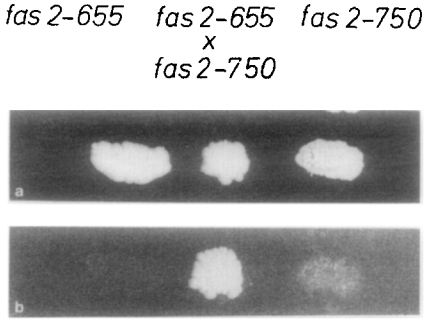


Fig. 1. In vivo complementation between fas 2-750 and fas 2-655. The haploid mutants fas 2-750, a and fas 2-655, α as well as a mating mixture of both were grown on fatty acid containing solid complete media (9). After 1 day incubation at 30°C the colonies (a) were replica-plated onto fatty acid-free media and incubated there for another 2 days at 30°C (b).

TABLE 1: In vivo complementation between fas 2-750 and fas 2-655 fatty acid synthetase mutants.

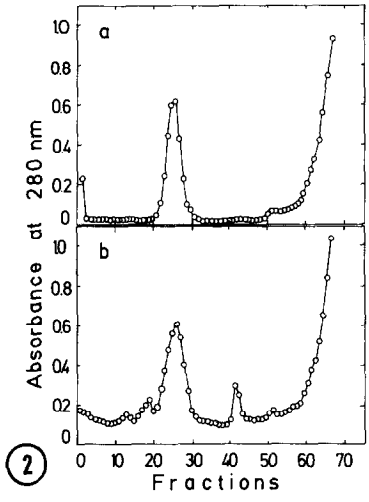
For experimental details see Materials and Methods.

enzyme function	relative specific activities				
	fas 2-750	fas 2-655	fas 2-750 + fas 2-655 (1:1, w/w, untreated mixture)	fas 2-750 + fas 2-655 (1:1, w/w, diss.-reass. mixture)	fas 2-750 x fas 2-655 (from the diploid)
	% of wild type				
overall fatty acid synthesis	-	-	-	-	44
acetyl transfer	158	115	136	143	102
malonyl transfer	254	157	203	222	232
palmityl transfer	342	205	275	300	266
enoyl reduction	163	115	137	220	87
dehydratation	262	170	215	195	134
ß-ketoacyl synthesis	-	-	-	-	51
ß-ketoacyl reduction	116	132	124	124	164
[^{14}C]-pantothenic acid incorporation	-	-	-	-	54

Other than *in vivo*, however, the hybrid enzyme constructed *in vitro* by dissociation and subsequent reassociation of a mixture of the two pantetheine-free mutant fatty acid synthetases remains enzymatically inactive (Table 1). This is expected since this enzyme, as a product of two pantetheine-less mutant synthetases, must remain pantetheine-free under the experimental conditions. Nevertheless, according to its *in vivo* behaviour the pantetheine-free *fas 2-750/fas 2-655* hybrid enzyme synthesized *in vitro* should theoretically, under appropriate conditions, bind 4'-phosphopantetheine. Therefore, this apoenzyme should be a suitable substrate for the enzyme transferring phosphopantetheine to the yeast fatty acid synthetase complex.

Interestingly, when the subunits of pantetheine-free fatty acid synthetases are reassociated to the intact complex, usually additional association products are observed besides the normal hexameric aggregate. Upon sucrose density gradient centrifugation of the reassociation mixture, 1-2 minor components migrating faster than the main peak (Fig. 2b) were regularly observed although slower migrating components occured in some cases, too. All additional components shown in Fig. 2b had essentially the same specific ß-ketoacyl reductase activities as the enzyme in the main peak. A similar heterogeneity of the association products was never observed with the wild type enzyme nor with any pantetheine-containing *fas*-mutant fatty acid synthetase so far studied under the same conditions (Fig. 2a). The physiological importance of this effect of pantetheine on proper complex association remains unclear as long as it is not known whether *in vivo* pantetheine is bound to the isolated α-subunits or to the $\alpha_6\beta_6$ apoenzyme.

Identification of coenzyme A: fatty acid synthetase apoenzyme 4'-phospho-pantetheine transferase activity in yeast. Using the activation of *in vitro* synthesized pantetheine-free *fas 2-750/fas 2-655* hybrid fatty



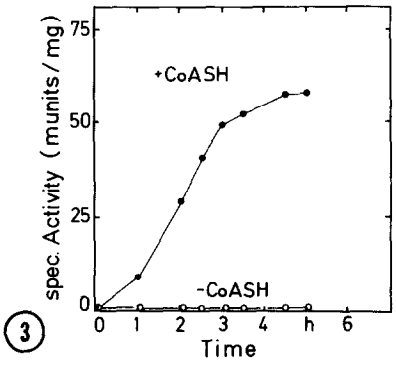


Fig. 2. Sucrose density gradient profiles of hybrid fas 2-1322/fas 2-2562 apoenzyme (b) and reassociated wildtype fatty acid synthetase (a). Dissociation and subsequent reassociation of wildtype and mutant fatty acid synthetase subunits was performed as described in Fig. 3. The reassociated synthetases were subject to 10-30 % sucrose density gradient centrifugation for 150 min at 45.000 upm in a Sorvall TV 850 vertical rotor using a Spinco Model L 5.65 ultracentrifuge equipped with a slow acceleration accessory (Beckman).

Fig. 3. Activation of fas 2-750/fas 2-655 hybrid fatty acid synthetase apoenzyme to the corresponding holoenzyme.
The hybrid apoenzyme was prepared by dissociating and subsequently reassociating (7) a mixture of 3 mg of each of the purified fas 2-750 and fas 2-655 mutant fatty acid synthetases. After purification by sucrose density gradient centrifugation 0.8 mg of the apoenzyme were incubated at 25°C in a total volume of 1.0 ml with 100 nmoles of coenzyme A (if indicated), 100 umoles of phosphate buffer pH 7.4, 100 umoles of $MgCl_2$, 100 umoles of dithiothreitol and 12 mg of a partially purified extract of fas 1-3410. After incubation of the reaction mixture for the time periods indicated aliquots were tested for overall fatty acid synthetase activity. The fas 1-3410 extract was prepared by disrupting the yeast cells with glass beads and by centrifugation of the crude homogenate for 20 min at 25.300 xg and subsequently, for 30 min at 100.000 xg. To the resulting supernatant 21.9 g/100 ml of solid ammonium sulfate were added. After centrifugation, the precipitate was dissolved in a small volume of 0.1 M potassium phosphate buffer pH 7.4 containing 10^{-3} M ethylene diamine tetraacetate and 10^{-3} M dithiothreitol. Before use, this solution was dialyzed for 3 hr against the same buffer.

acid synthetase as an assay system, the presence of coenzyme A-dependent apoenzyme 4'-phosphopantetheine transferase activity could be demonstrated in a partially purified yeast cell extract (Fig. 3). To eliminate the background of endogeneous fatty acid synthetase activity, the yeast strain used for this experiment was fas-mutant deficient in any enzymatically or immunologically detectable fatty acid synthetase. The transferase activity observed was depending only on coenzyme A (Fig. 3),

while ATP had no effect on either the rate or the extent of holoenzyme formation. As expected, no activation at all was observed with either one of the two homogeneous fas 2-750 or fas 2-655 fatty acid synthetases under the same conditions.

The results presented suggest that the mechanism of fatty acid synthetase holoenzyme formation in yeast could be similar to that of ACP biosynthesis in E.coli (4). However, the specific activity of the holoenzyme obtained in vitro was only about 20 percent compared to that of the in vivo-made enzyme. It remains to be shown whether this discrepancy results from a different mechanism of holoenzyme biosynthesis in vivo or from suboptimal experimental conditions so far applied. Possibly, these difficulties may be eliminated by using a more purified transferase preparation or by altering the proportion of the constituent subunits in the hybrid apoenzyme. It is expected that by the experimental approach outlined in this study it will be possibly to purify the coenzyme A: fatty acid synthetase apoenzyme phosphopantetheine transferase and, using purified transferase and apoenzyme preparations, to investigate the mechanism of phosphopantetheine incorporation into the yeast fatty acid synthetase complex in its details.

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