Yeast Fatty Acid Synthase: Structure to Function Relationship†

Nirpinder Singh, Salih J. Wakil,* and James K. Stoops*
Verna and Marrs McLean Department of Biochemistry, Baylor College of Medicine, Houston, Texas 77030
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ABSTRACT: The yeast fatty acid synthase is a multifunctional enzyme composed of two nonidentical subunits in an $\alpha_6\beta_6$ complex that is active in synthesizing fatty acids. The seven catalytic activities required for fatty acid synthesis are divided between the α and β subunits such that the $\alpha_6\beta_6$ complex has six complements of each activity. It has been proposed that these are organized into six centers for fatty acid synthesis. There are different opinions regarding the operation of these centers in the $\alpha_6\beta_6$ complex, one view being that they are functionally independent and the other proposes half-sites activity for the complex. We have attempted to distinguish between these proposals by the most direct method of active site titration, i.e., quantitation of fatty acyl product in the absence of turnover. This was accomplished by using *p*-nitrophenyl thioacetate and thiophenyl malonate (in place of the coenzyme A analogues) as substrates along with NADPH, thereby depriving the yeast synthase of coenzyme A required to release product as fatty acyl coenzyme A. The amount of fatty acyl product formed was quantitated by gas–liquid chromatography, as well as by direct estimation of radioactivity in the product when *p*-nitrophenyl thio[1-^{14}C]acetate was used as a substrate. In both cases, a stoichiometry of close to six was found for mole of fatty acid synthesized per mole of $\alpha_6\beta_6$ complex. This indicates that there are six functional centers for fatty acid synthesis in the multifunctional yeast $\alpha_6\beta_6$ fatty acid synthase and that these centers operate independently. It can also be deduced that coenzyme A does not play an essential role in the steps involved in catalysis of fatty acid synthesis as is the case for animal fatty acid synthase. However, the finding of full-site activity for the yeast synthase is in accord with our previous report of the identical behavior of chicken liver fatty acid synthase.

The fatty acid synthase of yeast (*Saccharomyces cerevisiae*) has been well established to be a multifunctional protein. Evidence obtained from genetic and biochemical studies shows that the seven component catalytic activities required for fatty acid synthesis are distributed between two nonidentical subunits (Schweizer et al., 1978; Stoops & Wakil, 1978). These subunits, designated as α and β, were also shown to be encoded in two unlinked gene loci. Yeast fatty acid synthase purified to homogeneity is thus seen as two peptides in equal amounts on sodium dodecyl sulfate–polyacrylamide gel electrophoresis, with M_r of 212 000 (α) and 203 000 (β) (Stoops et al., 1978a). The molecular weight of the active complex of yeast fatty acid synthase was determined to be 2.4×10^6, indicating that this complex is an $\alpha_6\beta_6$ oligomer (Stoops et al., 1978a).

The catalytic activities associated with the α subunit are known to be β-ketoacyl synthase, β-ketoacyl reductase, and the prosthetic group 4′-phosphopantetheine; the remaining five catalytic activities, i.e., acetyl and malonyl transacylase, palmitoyl transferase, dehydratase, and enoyl reductase, are associated with the β subunit (Schweizer et al., 1978; Stoops & Wakil, 1978). Estimates of the pantetheine content of yeast fatty acid synthase range between 3.5 and 6.0 mol/mol of synthase (2.4×10^6 dalton complex) (Schweizer et al., 1970; Stoops et al., 1978a). The investigations utilizing chemical modification reagents specific for the β-ketoacyl synthase activity also indicate the presence of six complements of this activity in the synthase $\alpha_6\beta_6$ complex (Stoops & Wakil, 1980). FMN is an essential cofactor in the enoyl reductase activity, and it is known that approximately 5 mol of FMN are bound per mole of synthase ($\alpha_6\beta_6$) (Oesterhelt et al., 1969). From these findings, it was deduced that the native form of the synthase complex possesses nearly 6 equiv of each catalytic activity required for fatty acid synthesis.

Electron microscope visualization has led to the proposal of a model for the $\alpha_6\beta_6$ complex that depicts an ovate structure that has on its short axis six abutted α subunits with the β subunits arranged on either side (Stoops et al., 1978a). Chemical modification studies using the bifunctional probe 1,3-dibromopropanone led to the discovery that the active center of the β-ketoacyl synthase contains two juxtapositioned, essential thiols derived from neighboring α subunits (Stoops & Wakil, 1981). Thus, two half α subunits together with one β subunit have all the activities required for fatty acid synthesis, and therefore, there are potentially six fatty acid synthesizing centers in the synthase $\alpha_6\beta_6$ complex.

Chemical modification reagents found to be specific for the various partial activities of the complex have been shown to react with each target site independently, thereby indicating the absence of cooperative interactions. One exception was a report that iodoacetamide, which specifically modifies an active cysteine SH in the β-ketoacyl synthase site, reacts with only three of the six possible sites on an $\alpha_6\beta_6$ complex (Oesterhelt et al., 1977). It was then proposed that the synthase is half-sites active due to negative cooperativity in the operation of the condensing enzyme. However, in a separate investigation it was found that the reagent modifies six cysteine residues with no evidence of negative cooperativity (Stoops & Wakil, 1981).

These lines of evidence that led to opposing conclusions have, together with some genetic evidence, spawned two different models for the function of the synthase complex. One describes a half-sites active synthase with conformationally induced noncooperativity (Schweizer, 1984) and the other a full-sites active enzyme with each fatty acyl synthesizing center oper-

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ating independently (Stoops & Wakil, 1981). The evidence used in support of these opposing viewpoints is not convincing; consequently, we have performed experiments to determine the stoichiometry of product synthesized by the enzyme as the most direct means to distinguish between half- and full-site reactivity (Ladzunski, 1974).

Experimental Procedures

Preparation and Assay of Enzyme. Yeast fatty acid synthase was prepared and assayed as described previously (Stoops et al., 1978a) and had a specific activity of approximately 3000 nmol of NADPH oxidized min^{-1} mg^{-1} at 25 °C. The protein concentration was estimated with a Gilford 240 recording spectrophotometer and the relationship $A^{1\%}_{1cm} = 11.5$ at 280 nm (Stoops et al., 1978a). The wavelength and absorbance scales of the instrument were calibrated as described previously (Stoops et al., 1978b).

Chemicals. Buffers were prepared from Baker reagent-grade salts, and pH was determined as described previously (Stoops et al., 1983). NADPH and acetyl- and malonyl-CoAs[1] were purchased from P-L Biochemicals. 4-Nitrothiophenol was from Aldrich, acetic anhydride from Baker, and [1-^{4}C]-acetic anhydride from New England Nuclear. Radioactivity was estimated by counting the samples in 0.4% Omnifluor (New England Nuclear) in toluene.

Synthesis and Purification of Substrate Analogues. (*A*) *p-Nitrophenyl thioacetate* was prepared by the procedure of Vatter et al. (1968). The product was purified by crystallization from chloroform–hexane followed by crystallization from 85 °C water. Light yellow needle-shaped crystals were obtained, mp 83.0–83.5 °C (lit. mp 81.5–82.0 °C; Vatter et al., 1968).

p-Nitrophenyl thio[1-^{14}C]acetate was also made by the procedure of Vatter et al. (1968) from [1-^{14}C]acetic anhydride. The *p*-nitrothiophenol used was purified by dissolution in aqueous NaOH (under an atmosphere of argon) and precipitation with HCl. In this case, the *p*-nitrophenyl thio[1-^{14}C]acetate product was dissolved in acetonitrile and purified by HPLC on a 1 × 25 cm, Whatman Partisil M9 column, chromatographed in toluene–hexane (75:25 v/v; flow rate 1.0 mL/min). The elution of product was monitored by absorbance at 287 nm. In this system, *p*-nitrophenyl thioacetate eluted distinct from *p*-nitrothiophenyl and *p*-nitrothiophenyl disulfide. The purified product was demonstrated to be pure by rechromatography in the same system.

Radiospecific activity of the product was determined by radioactive counting of an aliquot from a stock solution in acetonitrile and measurement of concentration by absorbance at 287 nm in 2% ethanol (λ_{max} in 2% ethanol = 287 nm, $\epsilon_{287} = 9.8 \times 10^3$; Raoult & Vilkas, 1968). The radiospecific activity thus calculated was 1.54 Ci/mol and was not seen to change over a period of several months when the substance was stored in an acetonitrile solution at liquid nitrogen temperature.

(*B*) *Thiophenyl malonate* was prepared by the procedure of Nakata (1957). The product was purified by crystallization from toluene–hexane, chloroform–hexane, and then toluene–hexane again, mp 74.3–74.8 °C (lit. mp 72–73 °C; Howard et al., 1965).

Yeast Synthase Reaction with Substrate Analogues. Synthase from yeast stored at −20 °C in 0.1 M potassium phosphate, 1 mM EDTA, 1 mM cysteine, pH 7.4, and 10% glycerol (v/v) in suspension in the presence of 90% ammonium sulfate was dialyzed against two changes of 1 L each of extensively argon-degassed 0.1 M sodium phosphate, 1 mM EDTA, pH 6.5, at 0 °C. All subsequent manipulations with the enzyme were carried out under positive argon pressure or in tubes sealed under argon pressure.

Stock solutions of phenyl thiomalonate, *p*-nitrophenyl thioacetate , or *p*-nitrophenyl thio[1-^{14}C]acetate were made in acetonitrile and stored at −20 °C. NADPH solutions were made in water immediately before use. For each reaction, a substrate solution was mixed in argon-degassed buffer of the same composition as the dialysis buffer. Final acetonitrile concentration in the substrate mixture was 3% (v/v) or less. To initiate the reaction, equal volumes of enzyme in buffer (approximately 5 mg/mL) and the substrate mixture were mixed. Final substrate concentrations were 0.15 mM NADPH, 1.0 mM phenyl thiomalonate, and 0.05 mM *p*-nitrophenyl thioacetate. At the specified time, aliquots of this reaction mixture were withdrawn and quenched in tubes containing sufficient alkali to raise the pH to >12 or treated with performic acid prepared as described previously (Stoops & Wakil, 1981). At this stage, an aliquot containing a known amount of $C_{17:0}$, $C_{19:0}$, and $C_{21:0}$ fatty acids in toluene was added to each of the alkali-treated samples meant for GLC analysis. Samples for extraction and counting of radioactivity were supplemented with excess $C_{16:0}$ and $C_{18:0}$ fatty acids to serve as carriers.

Extraction of Fatty Acids. At the end of the reactions, samples were treated with alkali in a boiling water bath for 30 min to hydrolyze all fatty acid esters. Performic acid hydrolyses were carried out at 0 °C for 2 h to cleave all fatty acyl thio esters. Fatty acids were then extracted 3 times by employing the Bligh–Dyer procedure (Bligh & Dyer, 1959).

Quantitation of Radioactivity in Fatty Acids. Chloroform extracts of samples prepared from reaction mixtures containing *p*-nitrophenyl thio[1-^{14}C]acetate were evaporated under nitrogen and redissolved in 2 mL of hexane. The hexane solutions were mixed with 2 mL of 0.1 N HCl. The hexane phase of each sample was transferred to a scintillation vial, pooled with a second hexane extract of the aqueous phase, evaporated under nitrogen, redissolved in 0.4% Omniflour, and counted.

Methylation of Fatty Acids and Chromatography of Fatty Acid Methyl Esters. Methyl esters of fatty acids were prepared by treatment with diazomethane as described previously (de Boer & Backer, 1954; Schlenk & Gellerman, 1960). The esters were purified by thin-layer chromatography on silica gel (HR) plates (20 × 6 cm), developed in chloroform. The spots containing methyl esters were scraped off, and the esters were eluted from the gel with hexane–diethyl ether (90:10 v/v). The solvents were then evaporated, and the esters were dissolved in carbon disulfide prior to analysis by gas–liquid chromatography on a Varian 3700 gas chromatograph equipped with a FID and a 180 cm × 2 mm (i.d.) glass column packed with 10% SP-2330 on 100/120 Chromosorb WAW (Supelco). Carrier gas was helium with a flow rate of 40 mL/min, and the column was programmed from 140 to 220 °C at 2 °C/min. Injector temperature was 230 °C, and the detector was maintained at 240 °C. The fatty acid methyl ester peaks were identified by matching retention times with those of peaks from a known standard run under the same conditions. The area under each peak was integrated on a Columbia Scientific Industries Supergrator-3 programmable integrator and converted to relative nanomoles by comparison with the peak areas of the internal standards.

[1] Abbreviations: CoA, coenzyme A; EDTA, ethylenediaminetetraacetic acid; FID, flame ionization detector; GLC, gas–liquid chromatography; HPLC, high-pressure liquid chromatography.

Table I: Quantitation of Product Formed Using *p*-Nitrophenyl Thio[1-^{14}C]acetate

synthase prepn	synthase in reaction (nmol)[a]	reaction time (min)	radioactivity in product (dpm)[b]	fatty acyl product (nmol)[c]	fatty acyl product/ synthase (mol/mol)
I	0.20	40	4926	1.34	6.8
II	0.26	20	4219	1.23	4.7
III	0.29	40	5465	1.59	5.3

[a] Calculated using a molecular weight for $\alpha_6\beta_6 = 2.4 \times 10^6$ and $A^{1\%}_{1cm} = 11.5$ at 280 nm. [b] Average of three separate determinations; blanks in each case were 30–50 dpm. [c] Calculated using a radiospecific activity of product = 1.54 Ci/mol. See Experimental Procedures.

Analyses of radioactive methyl ester samples were carried out with a Hewlett-Packard 57110 A gas chromatograph on a similar column as mentioned above. Carrier gas in this case was nitrogen at 40 mL/min. The instrument was equipped with a splitter device, which was adjusted to channel approximately 15% of the effluent gas to the FID for determination of mass and the remaining to an exit port where samples were collected for measuring radioactivity. These samples were counted in toluene–Omnifluor as described before (Singh et al., 1984).

Results

Out experiments were designed to quantitate fatty acyl product formed by the native yeast synthase $\alpha_6\beta_6$ complex in the absence of product turnover. To achieve nonturnover conditions, we deprived the synthase of free coenzyme A required as a nucleophilic acceptor by the chain-terminating palmitoyl transferase activity. Under normal assay conditions allowing turnover, no coenzyme A need be added, since that which is released by transacylation from acetyl- and malonyl-CoAs suffices. In our titration experiments we replaced acetyl- and malonyl-CoAs with *p*-nitrophenyl thioacetate and thiophenyl malonate. These substrate analogues have previously been shown to be transacylated by the enzyme to coenzyme A and were found to be competent in allowing fatty acid synthesis albeit at a much slower rate (Singh et al., 1985). Over the time period chosen for the experiment, turnover due to fatty acid release by nonspecific nucleophiles was negligible, permitting an accurate estimate of the stoichiometry of product formed. Since the carbon atoms from the acetyl substrate appear only once in the fatty acyl product, we used *p*-nitrophenyl thio[1-^{14}C]acetate to directly quantitate total fatty acyl product formed. Also, gas–liquid chromatographic analyses were performed on samples from reactions containing nonradioactive and radioactive *p*-nitrophenyl thioacetate for an independent measure of total product and the distribution of product chain length.

Quantitation of Fatty Acyl Product Using p-Nitrophenyl Thio[1-^{14}C]acetate. The data presented in Table I show the incorporation of [^{14}C]acetate into fatty acids and the relative amounts of products synthesized with different enzyme preparations and times of incubation. Three different preparations of synthase were employed, and in each case the stoichiometry of product formed was close to the maximum expected value of 6. The formation of product appears to be substantially the same after 20 and 40 min of reaction, indicating the enzyme did not turnover the fatty acyl product.

The fatty acyl product formed under these nonturnover conditions would most likely remain in thio ester linkage to the 4′-phosphopantetheine of the enzyme (Schreckenbach et al., 1977). In order to determine whether that was the case, we subjected samples from a radioactive assay to performic acid oxidation, after completion of fatty acid synthesis. Unlike alkaline hydrolysis, this treatment would only release fatty acyl moieties in thio ester linkage. A comparison of the data from quantitation of radioactivity released by alkaline hydrolysis vs. performic acid oxidation in Table II shows that 70–80% of fatty acyl product is in thio ester form.

Quantitation and Chain Length of Fatty Acyl Product Formed. Gas–liquid chromatographic analyses of fatty acid methyl esters generated from the product of yeast fatty acid synthase are shown in Figure 1. Panels A and B show the fatty acids present in extracts obtained from reaction mixture alone and with enzyme alone, respectively, illustrating the background levels of fatty acids expected in the analysis. Panels C and D show the results of analyses of fatty acids obtained from reaction mixtures after incubation for 20 and 40 min, respectively. Quantitation of the methyl esters of the fatty acids synthesized de novo was performed by comparison of peak areas with internal standard fatty acid methyl ester peaks. The results of such determinations are shown in Table III, after correction for endogenous fatty acid content.

The relative amount of each fatty acid synthesized varies substantially with time of incubation. After 20 min of incubation, the major product was $C_{18:0}$, while after 40 min the longer chain fatty acids $C_{20:0}$ and $C_{22:0}$ predominated. However, the total stoichiometry of fatty acids synthesized remains the same at each time period and is very near (within 8%) the maximum possible value of 6 mol/mol of synthase ($\alpha_6\beta_6$). These results further support the contention that the enzyme did not release fatty acids because of the CoA requirement for the terminal reaction.

The variation in the chain length of the fatty acids synthesized was confirmed at each time period by collecting and counting the radioactivity of fractions from gas–liquid chromatography of samples labeled with *p*-nitrophenyl thio[1-^{14}C]acetate (Table IV). This method is not as quantitative as determination of mass by relation to internal standard fatty acids, due to difficulty in trapping all of the fatty acid methyl ester in each peak. However, the distribution of radioactivity obtained is a fair indication of the array of de novo fatty acids

Table II: Comparison of Radioactivity in Fatty Acyl Esters Released by Alkaline Hydrolysis vs. Performic Acid Oxidation[a]

expt	mode of hydrolysis	synthase in reaction (nmol)	reaction time (min)	radioactivity in product (dpm)	ratio of radioactivity of performic/alkaline hydrolysis
I	alkaline hydrolysis	0.20	40	4580	0.70
	performic acid	0.20	40	3223	
II	alkaline hydrolysis	0.26	20	4219	0.82
	performic acid	0.26	20	3445	

[a] See Experimental Procedures.

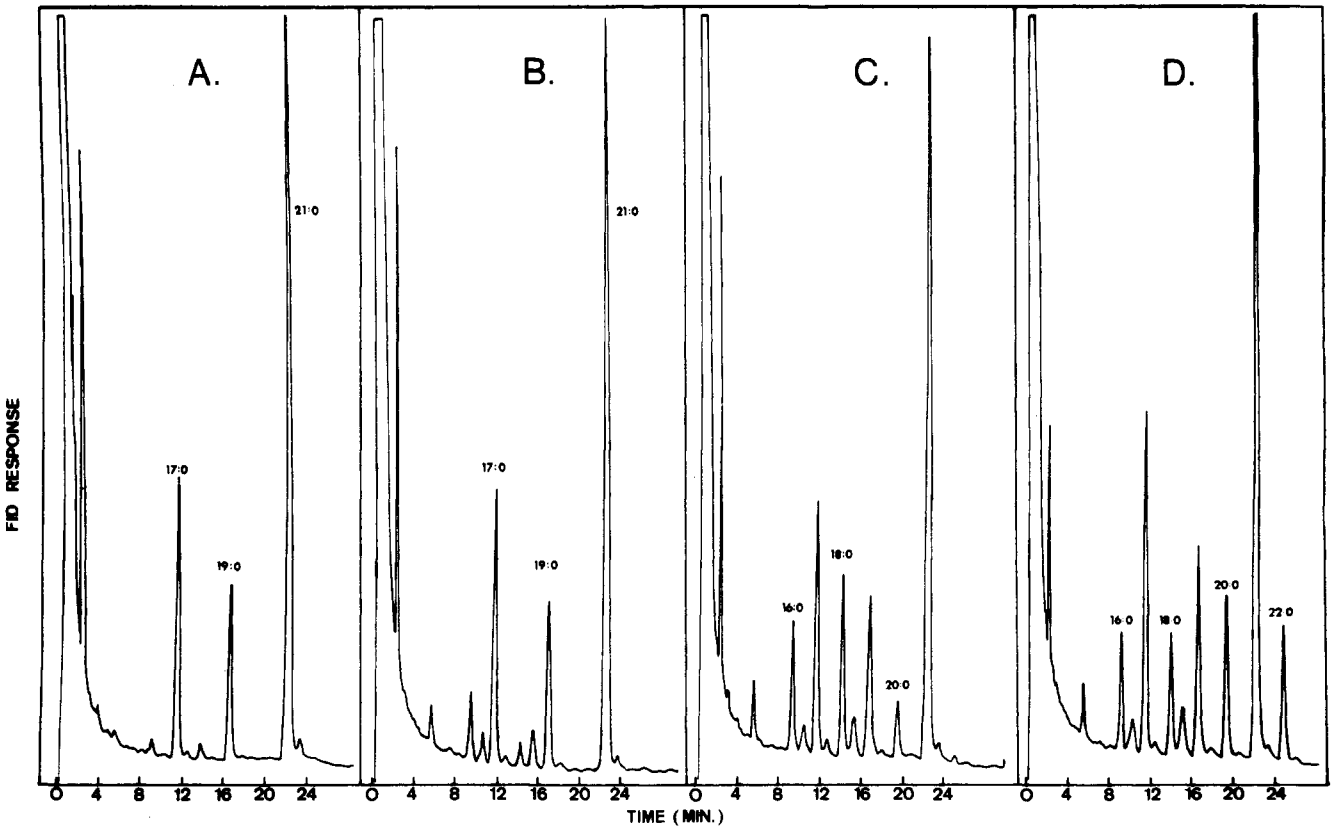

FIGURE 1: Comparison of endogenous and de novo synthesized fatty acids. Gas–liquid chromatography of fatty acids extracted from enzyme buffer and substrate mix (A), from 1.15 mL of synthase (2.96 mg/mL) mixed with an equal volume of buffer (B), or from 1.15 mL of the same synthase mixed with 1.15 mL of substrate mix after 20 min of reaction (C) and after 40 min of reaction (D). In each case the fatty acids $C_{17:0}$, $C_{19:0}$, and $C_{21:0}$ were added as internal standards.

Table III: Stoichiometry of Fatty Acids Synthesized As Determined by GLC Analyses[a]

reaction time (min)	fatty acids	fatty acids synthesized/synthase (mol/mol)[b]
20	14:0	0.18
	16:0	1.04
	18:0	3.19
	20:0	1.19
	22:0	0.10
	24:0	
		5.69[c]
40	14:0	
	16:0	0.18
	18:0	0.90
	20:0	2.51
	22:0	1.88
	24:0	0.05
		5.52[c]

[a] Calculated by normalizing amount of each fatty acid relative to the added internal standards $C_{17:0}$, $C_{19:0}$, and $C_{21:0}$. [b] Calculated from the amount of fatty acids synthesized by 1.42 nmol of $\alpha_6\beta_6$ yeast fatty acid synthase. [c] Total.

Table IV: Relative Amounts of Fatty Acyl Product from Gas–Liquid Chromatographic Analysis

	% of total fatty acids synthesized as determined by			
	mass		radioactivity	
fatty acid	20 min	40 min	20 min	40 min
$C_{12:0}$			3	2
$C_{14:0}$	3		7	5
$C_{16:0}$	18	3	13	7
$C_{18:0}$	56	16	43	20
$C_{20:0}$	21	46	26	36
$C_{22:0}$	2	34	8	26
$C_{24:0}$		1		5

formed. A comparison of product distributions obtained by these two methods shows that the conclusion regarding the major product species is the same in each case (cf. Table IV).

Discussion

It is apparent from the data in Table I that the thiophenyl derivatives of acetate and malonate used are indeed substrates for the synthesis of fatty acids by the yeast synthase. The incorporation of radioactivity into an organic-extractable fraction is indicative of de novo synthesis. The calculated stoichiometry of product determined by direct radioactive counting of a Bligh and Dyer extract, at 20 and 40 min of reaction and with three different enzyme preparations, is near the maximum possible 6 mol of product/mol of $\alpha_6\beta_6$, thereby falling within the range expected for a full-sites active synthase. The stoichiometry also remains relatively constant with time of reaction and a 1.5-fold variation in the amount of enzyme used, indicating saturation of the fatty acyl synthesizing sites.

The data in Table II indicate that not all of the fatty acyl product bound to the enzyme may be in thio ester form, although the majority (70–80%) is. For the most part the bound product is thus likely to be a thio ester of the 4′-phosphopantetheine or the β-ketoacyl synthase site active cysteine.

The quantitation of fatty acyl product by gas–liquid chromatography as illustrated in Figure 1 affords much greater accuracy due to the presence of internal standards during all handling steps, thus automatically correcting for losses suffered. It can also be ascertained from the relative recovery of internal standards added that there have been no preferential losses of fatty acids in the range of the chain length of interest.

As seen from Table III, the quantitation of total amount of product by this method after 20 and 40 min of reaction

yields values that correspond very closely (within 8%) to those expected for a full-sites active enzyme. It is also apparent that the synthase inhibited from turning over product slowly elongates the bound fatty acyl moieties. It is probable that if a short enough reaction time were chosen, the first species seen to accumulate would be $C_{16:0}$, which is the major product under conditions of turnover. The principle pattern of this distribution is reaffirmed by the results in Table IV, comparing the distribution of fatty acids obtained by mass analyses with that obtained by determination of radioactivity in each peak collected from the gas–liquid chromatograph.

It is known that depletion of free coenzyme A from the reaction inhibits yeast fatty acid synthase (Lynen, 1980) and also that a high concentration of coenzyme A is inhibitory (Schweizer, 1984). Our experiments suggest that these effects are most likely to occur at substrate binding or product release levels, and that involvement of coenzyme A as an integral species in the intermediate steps of fatty acid synthesis is not likely. This situation is in contrast to the animal synthases (Stern et al., 1982), where coenzyme A is thought to be essential for activity because of its involvement in correcting misacylation due to a random substrate loading process.

Our results do not support the model suggested for a half-sites active synthase (Oesterhelt et al., 1977; Schweizer, 1984) but favor the one for a full-sites active $\alpha_6\beta_6$ complex (Stoops & Wakil, 1981). Thus, in an all or none phenomenon we see no evidence of noncooperative interactions leading to less than full-sites activity. It should also be pointed out that the half-sites reactivity reported for iodoacetamide has since been contradicted (Stoops & Wakil, 1981) and may be explained by aberrant behavior due to a partially inactivated or less active synthase.

The current full-sites activity reported for yeast synthase is in harmony with our earlier report of similar behavior of the homodimeric chicken liver synthase, which has two potential fatty acyl synthesizing centers in each native α_2 complex (Singh et al., 1984). All known multifunctional enzymes are oligomeric, and in the two instances noted above, this higher order structure does not seem to be reflected in an exhibition of cooperativity between synthesizing centers.

Finally, it should be noted that the artificial substrates *p*-nitrophenyl thioacetate and *p*-nitrophenyl malonate are well suited for a spectrophotometric study of transacylation processes catalyzed by the synthase. It is thus possible to determine—with a rigor not previously attainable—the kinetics of acylation and deacylation of the enzyme and the order of substrate loading steps. Investigation along these lines is currently in progress in our laboratory.

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