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Direct Desaturation of Free Myristic Acid by Hen Liver Microsomal Δ^9-Desaturase without Prior Activation to Myristoyl-CoA Derivative

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Direct desaturation of free myristic acid by hen liver microsomal Δ^9-desaturase without prior activation to myristoyl-CoA by the addition of adenosine triphosphate (ATP) and CoA was observed when the incubation medium was mixed at mixing speeds of >250 rpm in the presence of fatty acid-binding proteins (FABP). Desaturation was linear with time and proportional to the microsomal protein concentration. Desaturation was maximal at pH 7.9. The greatest desaturation rate was observed at a mixing speed of 500 rpm in the presence of FABP. Desaturation decreased at mixing speeds of >500 rpm. Data suggest that when myristic acid is bound to FABP in the form of protein−monomer complexes, its activation to the CoA derivative is not necessary for it to be desaturated by the Δ^9-desaturase when using mixing rates of >250 rpm. Myristic acid−FABP complexes serve as substrates for the Δ^9-desaturase at mixing rates of >250 rpm. Desaturation was reduced by bovine serum albumin and α-bromohexadecanoate, and no desaturation was observed in the absence of FABP. These findings suggest that FABP may regulate the accessibility of fatty acids in the desaturation reaction to the active site of the desaturase rather than just protect the membrane-bound desaturase from the cytotoxic effect of free fatty acids.

KEYWORDS: Myristoleoyl-CoA; desaturation; enzymatic reaction; fatty acid-binding protein; free myristic acid

INTRODUCTION

Enzymatic desaturation of saturated fatty acids to form monounsaturated fatty acids is known to occur in various aerobic organisms including yeast and several animal species (*1*). Early experiments using liver homogenates established that the desaturation of fatty acids involves two enzymatic reactions, the catalyzed activation of the fatty acid to the acyl-CoA derivative by thiokinases in the presence of CoA, ATP, and Mg^{2+} followed by desaturation of the derivative by the acyl-CoA desaturase system (*2*).

It has been reported that mammalian Δ^9-fatty acyl-CoA desaturases are bound to the endoplasmic reticulum and have an obligatory requirement for reduced nicotinamide adenine dinucleotide (NADH), molecular oxygen, cytochrome b_5, and NADH cytochrome b_5 reductase (EC 1.6.2.2) (*3*). These components have been isolated and characterized by other investigators (*4*, *5*). Stearoyl CoA desaturase is completely submerged in the hydrocarbon region of the membrane with the catalytic domain exposed to the aqueous environment to permit reduction of its non-heme iron by cytochrome b_5 and interaction with the hydrophilic portion of substrate molecules (*6*).

Using purified stearoyl-CoA desaturase, Enock et al. (*6*) reported that 9,10-desaturation of fatty acyl-CoA derivatives with chain lengths of 12−19 carbon atoms is catalyzed by the same enzyme, with stearoyl-CoA being the best substrate. These experiments demonstrated the lack of desaturation of lipid-bound stearate and established fatty acyl-CoA as the true substrate for stearoyl CoA desaturase (7). The same has since been demonstrated to be true for the Δ^6- and Δ^5-fatty acyl CoA desaturases (*8*).

The first evidence suggesting the possibility of a direct desaturation of oleoyl phosphatidycholine to linoleoyl phosphatidylcholine by a membrane-bound enzyme system was provided by Gurr et al. (*9*), whereas Pugh and Kates (*10*) also provided evidence for direct Δ^5-desaturation of eicosatrienoyl lecithin to arachidonoyl lecithin by rat liver microsomes.

We have undertaken studies to determine whether free myristic acid could be directly desaturated without first being activated to the CoA derivative. This question has previously been considered by other investigators (*2*, *7*, *11*−*16*), but no evidence for the direct desaturation of free fatty acids without being first activated to the CoA derivative has been presented. The data presented here will clearly show direct desaturation of free myristic acid in the absence of CoA and ATP, when the incubation medium is mixed at speeds of >250 rpm in the presence of fatty acid-binding protein (FABP). Several properties

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of the enzyme system will be studied including specific activities, substrate specificity, and interaction of substrate with BSA and FABP.

MATERIALS AND METHODS

Materials. Tetradecanoic, *cis*-9-tetradecenoic, pentadecanoic acids, bovine serum albumin (BSA), trypsin, trypsin inhibitor, sodium lactate, α-bromohexadecanoate, NADH, and oxidized nicotinamide adenine dinucleotide (NAD) were obtained from Sigma Chemical Co. (St. Louis, MO). The standards were of >98% chemical purity according to the manufacturers. Magnesium chloride and potassium hydroxide were obtained from Aldrich Chemical Co. (Milwaukee, WI). A Lightnin Lab Master (SI) mixer (model L1U03 and an impeller [type A100 (diameter = 1 in.)] were obtained from Matoon and Lee Equipment (Farmington, MI). Laying hens (White Leghorn) were obtained from the Michigan State University Poultry Farm (East Lansing, MI).

Preparation of Hen Liver Microsomes. For each set of experiments, three laying White Leghorn hens were sacrificed. The whole livers were excised and cooled immediately over ice. The livers were minced and mixed together to decrease possible variations in desaturation yield from one single-liver preparation to another. A 20 g sample of the ground livers was immediately homogenized in a Waring blender for 30 s with 60 mL of chilled 0.1 M potassium phosphate buffer (pH 7.4) containing 0.3 M sucrose. The temperature of the buffer solution was 0−2 °C. The resulting homogenate was centrifuged at 15000*g* for 30 min at 2 °C. The sedimented cellular debris and mitochondria were discarded. Microsomes containing the Δ^9-desaturase system were either used directly in the enzyme assay as a mitochondrial supernatant or sedimented from the postmitochondrial supernatant fraction by centrifugation at 105000*g* for 1 h at 2 °C. The resulting microsomal pellet was suspended either in the homogenizing buffer or in the microsomal supernatant (containing the cytoplasmic proteins) to provide a final concentration of 2.66 mg of microsomal protein/mL. The protein concentration was determined according to the method of Lowry et al. (*17*). The remaining ground livers were stored at −20 °C but for no longer than 10 h. Independent studies indicated that there was no loss in enzymatic activity over the duration of the holding period.

Enzyme Assays. Except where indicated otherwise, the incubation mixture with BSA-complexed myristic acid was 60 mg of BSA, 133 μM free C14:0 (BSA and C14:0 were incubated at room temperature for 5 min prior to the addition of the enzyme system), 1.16 mM NADH, 1.31 mM $MgCl_2$, hen liver mitochondrial supernatant containing 8 mg of microsomal protein and 94.5 mg of cytosolic protein, 40 mM potassium phosphate (pH 7.4), 120 mM sucrose (pH 7.4), and distilled water to a total volume of 7.5 mL. The digest was mixed at 500 rpm for 30 min at 37 °C in a 50 mL glass beaker.

Lipid Extraction and Fatty Acid Methyl Esterification. The enzymatic reaction was stopped by adding 20 mL of hexane/2-propanol (3:2, v/v). Samples were extracted three times with a mixture of hexane/2-propanol (3:2, v/v) and the combined solvent extracts evaporated under nitrogen. The internal fatty acid standard C15:0 (0.02 mg) was added to each of the samples, and the inherent fatty acids were then derivatized to their methyl esters according to the boron trifluoride/methanol method of Morrison and Smith (*18*).

Gas Chromatographic (GC) and GC−Mass Spectrometric (GC-MS) Analyses. GC analyses were carried out on a Hewlett-Packard 5890 A gas chromatograph (Avondale, PA) equipped with a flame ionization detector (FID), split mode (1/12), and a fused silica capillary column (30 m × 0.25 mm) coated with a 0.25 μm film of DB-225 (J&W Scientific, Folsom, CA). Operating conditions of the gas chromatograph were as follows: injector temperature, 275 °C; detector temperature, 300 °C; column temperature, 165 °C; hold for 5 min; increase oven temperature at 0.5 °C/min to 180 °C, hold for 20 min, increase at 10 °C/min to 215 °C, hold for 10 min. Fatty acid methyl esters were identified by comparing their relative GC retention times with those of known standards. Standard curves were generated from known concentrations of pure methyl ester standards of C14:0, *cis*-9-C14:1 ranging from 7.8 to 62.5 pg and containing 0.01 pg of C15:0. The peak areas corresponding to C14:0, *cis*-9-C14:1, and C:15:0 were determined using a digital integrator. The C14:0 and *cis*-9-C14:1 peak areas were divided by the internal standard area to obtain the response ratios for both standards and samples. The standard response ratios for C14:0 and *cis*-9-C14:1 were plotted against their corresponding concentrations. Standard response ratio plots should bracket the sample response ratio. The C14:0 and *cis*-9-C14:1 concentrations in the sample extracts were quantified from the standard response ratio plots and corrected for the dilution factor (df = 2). Initial analysis indicated that there was no endogenous *cis*-9-C14:1 in the medium. Thus, all of the *cis*-9-C14:1 observed was derived as a result of the desaturation reaction per se.

The identity of *cis*-9-C14:1 was confirmed by comparing the mass spectra of authentic *cis*-9-C14:1 standard and that derived from the desaturation of C14:0. The GC-MS analyses were carried out on a JEOL A × 505H double-focusing mass spectrometer equipped with a Hewlett-Packard 5890 J GC. The fatty acid methyl esters, dissolved in hexane, were injected using a splitless injection technique onto a 30 m × 0.25 mm i.d., 25 μm coated DB-225 capillary column interfaced directly into the ion source. The GC oven temperature was programmed from 100 to 165 °C at 5 °C/min and held for 5 min, then to 172 °C at 0.5 °C/min, and finally to 230 °C at 10 °C/min. The injector and transfer line temperatures were maintained at 230 and 230 °C, respectively. Fatty acids were analyzed in the *m*/*z* range of 45−600 for the methyl ester derivatives in positive electron ionization mode (EI).

Trypsin Digestion. To each 6 mL aliquot of the microsomal supernatant containing 31.5 mg/mL of cytosolic proteins was added 340 mg of trypsin, and the mixture was incubated for 1 h at 37 °C. Proteolysis was stopped with the addition of 680 mg trypsin inhibitor.

Statistical Analysis. All treatments were performed in duplicate ($n = 2$). The statistical analyses were based on the linear and polynomial regressions and analysis of variance (ANOVA) procedures of SYSTAT 7.0 (SPSS Inc., Chicago, IL). ANOVA was also used to analyze the data and included NADH, competitive inhibitors, heat treatment, trypsin digestion, BSA, and microsomal supernatant (MS) as main effects and BSA × MS as interaction. The Tukey multiple-comparison procedure was used for mean separation. If levels within a factor were significantly different, they were examined further using Student's *t* test for differences among individual means. Results were judged to be statistically significant on the basis of a type I error rate of 5%.

RESULTS AND DISCUSSION

Effect of Incubation Time. Under the assay conditions, the amounts of myristoleate formed were directly proportional to the incubation times. Incubations were stopped after 30 min, over which time the response of the enzyme was linear (**Figure 1**). Linearity of response did appear to be consistent for 60 min. The direct desaturation of free myristic acid was not affected by competing enzymic reactions.

Effect of Microsomal Protein Concentration. The rate of direct desaturation of free myristic acid was linear with microsomal protein concentrations over the range of 2−8 mg per 7.5 mL of final incubation volume (**Figure 2**). Inhibition occurred at protein concentrations >8 mg, with a marked decrease at the concentration of 10 mg per 7.5 mL of final incubation volume. A relatively high microsomal protein concentration of 8 mg per 7.5 mL of incubation volume was subsequently employed in all assays, unless otherwise indicated, to ensure there is enough production of myristoleate during the incubation time of the assay. At constant levels of substrate, monoene formation was inhibited at high protein concentrations. This finding was also observed when myristoyl-CoA was used as a substrate. The decrease in the desaturation rate with high protein concentrations could be due in part to the addition or accumulation of desaturation products.

Effect of pH. The pH of the reaction system influences the velocity of all enzyme-catalyzed reactions**.** The importance of pH in the desaturation reaction is due to its ability to (1) change the ionization state of the Δ^9-desaturase system, (2) change the

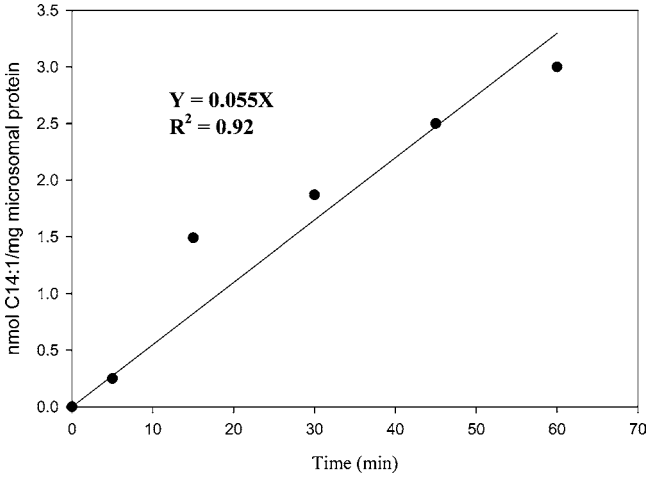

Figure 1. Direct desaturation of free myristic acid as a function of time by the Δ^9-desaturase system of hen liver microsomes. The complete system contained 8 mg of hen liver microsomal protein, 94.5 mg of cytosolic protein, 40 mM potassium phosphate (pH 7.4), 120 mM sucrose, 133 μM free C14:0, 60 mg of BSA, 1.16 mM NADH, and 1.31 mM $MgCl_2$ (pH 7.4), in a total volume of 7.5 mL. The digest was mixed at 500 rpm at 37 °C.

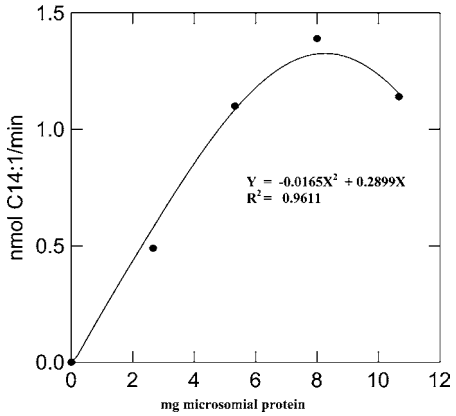

Figure 2. Direct desaturation of free myristic acid as a function of microsomal protein concentration by the Δ^9-desaturase system of hen liver microsomes. The complete system contained 94.5 mg of cytosolic protein, 40 mM potassium phosphate (pH 7.4), 120 mM sucrose, 133 μM free C14:0, 60 mg of BSA, 1.16 mM NADH, and 1.31 mM $MgCl_2$ (pH 7.4), in a total volume of 7.5 mL. The digest was mixed at 500 rpm for 30 min at 37 °C.

ionization state of the substrate, which in turn affects the reactivity of the reaction, (3) and change the solubility of the substrate solubilizing protein. The Δ^9-desaturase was active within a pH range 6.5−8.5 (**Figure 3**). The optimal pH for the formation of monoenes from the direct desaturation of free myristic acid in hen liver microsomes was 7.9.

Effect of Temperature. Temperature is a key factor in the desaturation reaction due to its ability to (1) change the rate of the reaction, (2) denature the desaturase system and the cytoplasmic proteins, and (3) change the fluidity and the crystalline−liquid crystalline phase transition of the membrane (*19*). The optimal temperature for the direct desaturation of free myristic acid by hen liver microsomes was 37 °C (**Figure 4**). At lower and higher temperatures, rates of the desaturation reaction were affected. No C14:1 formation was observed at 25 °C for the duration of the experiment (30 min). Mixing time has no effect on the temperature as the temperature remained unchanged during the whole incubation period.

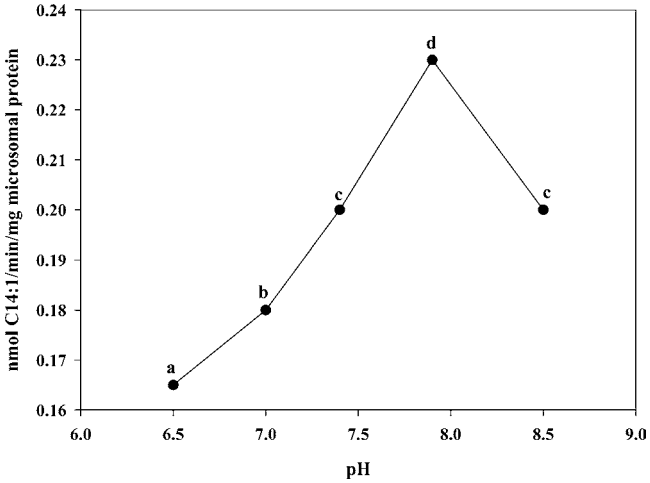

Figure 3. Effect of pH on the direct desaturation of free myristic acid by the Δ^9-desaturase system of hen liver microsomes. The complete system contained 8 mg of microsomal protein, 94.5 mg of cytosolic protein, 40 mM potassium phosphate (pH 7.4), 120 mM sucrose, 133 μM free C14:0, 60 mg of BSA, 1.16 mM NADH, and 1.31 mM $MgCl_2$, in a total volume of 7.5 mL. The digest was mixed at 500 rpm for 30 min at 37 °C. Means with no common letter are significantly different ($p < 0.05$).

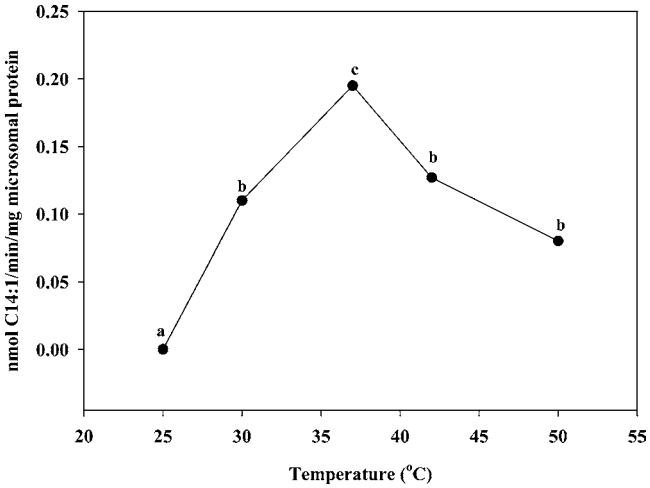

Figure 4. Effect of temperature on the direct desaturation of free myristic by the Δ^9-desaturase system of hen liver microsomes. The complete system contained 8 mg of microsomal protein, 94.5 mg of cytosolic protein, 40 mM potassium phosphate (pH 7.4), 120 mM sucrose, 133 μM free C14:0, 60 mg of BSA, 1.16 mM NADH, and 1.31 mM $MgCl_2$ (pH 7.4), in a total volume of 7.5 mL. The digest was mixed at 500 rpm for 30 min. Means with no common letter are significantly different ($p < 0.05$).

Effect of Reducing Agent. The generation of NADH in situ by adding NAD^+ and sodium lactate and taking advantage of the presence of lactate dehydrogenase (LDH) was investigated. LDH is present in the mitochondrial supernatant of hen liver (*16*). The effect of NADH and generated in situ NADH on the direct desaturation of myristic acid is shown in **Table 1**. The difference between added NADH and that generated in situ was not statistically significant. In subsequent experiments, NADH was generated in situ.

Effect of BSA. The capacity of BSA for tight binding of long-chain fatty acids is well-known. Studies of the structural interactions between FFA and BSA using ^{13}C NMR spectroscopy revealed multiple binding sites, hydrophobic interaction with the hydrocarbon chain, and electrostatic interaction with the carboxylate anion (*20*−*22*). Methyl esterification of the carboxyl group of long-chain fatty acids decreased the binding

Table 1. Effect of Pyridine Nucleotide on the Direct Desaturation of Free Myristic Acid by the Δ^9-Desaturase System of Hen Liver Microsomes[a]

pyridine nucleotide	C14:1[b] (nmol/min/mg of microsomal protein)
$NADH^1$	0.19a
$NADH^2$	0.18a

[a] The complete system contained 8 mg of microsomal protein, 94.5 mg of cytosolic protein, 40 mM potassium phosphate (pH 7.4), 120 mM sucrose, 133 μM free C14:0, 60 mg of BSA, 1.24 mM NAD, 3.09 mM sodium lactate, and 1.31 mM $MgCl_2$ (pH 7.4), in a total volume of 7.5 mL. The digest was mixed at 500 rpm for 30 min at 37 °C. $NADH^1$ was generated in situ, and $NADH^2$ was added directly. When NADH was added directly, NAD and sodium lactate were not added to the incubation medium. [b] Means with no common letter are significantly different ($p < 0.05$).

Table 2. Effect of Bovine Serum Albumin and Microsomal Supernatant on the Direct Desaturation of Myristic Acid by Hen Liver Microsomal Δ^9-Desaturase[a]

protein	C14:1[b] (nmol/min/mg of microsomal protein)
+BSA, +MS	0.137a
+BSA, −MS	0b
−BSA, +MS	0.156a
−BSA, −MS	0b

[a] The complete system contained 8 mg of microsomal protein, 94.5 mg of cytosolic protein, 40 mM potassium phosphate (pH 7.4), 120 mM sucrose, 133 μM free C14:0, 60 mg of BSA, 1.24 mM NAD, 3.09 mM sodium lactate, and 1.31 mM $MgCl_2$ (pH 7.4), in a total volume of 7.5 mL. The digest was mixed at 500 rpm for 30 min at 37 °C. The microsomal supernatant is the source of cytosolic proteins. +BSA indicates the presence of bovine serum albumin; −BSA indicates the absence of bovine albumin. +MS indicates the presence of microsomal supernatant; −MS indicates the absence of microsomal supernatant. [b] Means with no common letters are significantly different ($p < 0.05$).

of fatty acids to BSA by 40−50% at pH 7.4 (*13*). On the basis of **Table 2**, the decrease in the direct desaturation reaction was not significant in the presence of BSA. In the absence of the microsomal supernatant, direct desaturation of myristic acid did not occur whether BSA was present or not (**Table 2**). These observations might be attributed to the tight binding BSA−C14:0 complexes (multiple binding sites) (*20*, *22*, *29*) that make myristic acid sterically inaccessible to the active site of the Δ^9-desaturase. By contrast, when myristoyl-CoA was the substrate, BSA increased the Δ^9-desaturase specific activity by protecting the substrate from the competing enzyme present in the microsomal supernatant, but not from the Δ^9-desaturase (*23*).

Direct desaturation of myristic acid differs from that of myristoyl-CoA in that it requires not only a mixing speed of >250 rpm but also the microsomal fraction containing FABP. The presence of FABP in the cytosolic fraction of chicken liver has been reported (*24*), and the crystal structure of chicken liver basic FABP has been determined by X-ray crystallography (*4*).

Effect of Microsomal Supernatant. The omission of the microsomal supernatant containing FABP resulted in loss of desaturation activity regardless of whether BSA was present or not (**Table 2**). The microsomal supernatant was examined for protein that may be involved in the regulation of the direct desaturation of myristic acid using heat inactivation and trypsin digestion. The effect of heat inactivation and trypsin digestion on the capacity of microsomal supernatant to increase the desaturation activity revealed the involvement of such proteins. The microsomal supernatant is unstable to heat and undergoes major denaturation when boiled for 30 min. Boiling produced precipitation of the cytosolic proteins. The desaturase specific activity was completely lost as a result of heat precipitation of the cytosolic proteins (**Table 3**).

Table 3. Effect of Heat Treatment and Trypsin Digestion of the Microsomal Supernatant on the Direct Desaturation of Free Myristic Acid by the Δ^9-Desaturase System of Hen Hepatic Microsomes[a]

assay	C14:1[b] (nmol/min/mg of microsomal protein)
control	0.15a
heat	0b
trypsin	0b

[a] The complete system contained 8 mg of microsomal protein, 94.5 mg of cytosolic protein, 40 mM potassium phosphate (pH 7.4), 120 mM sucrose, 133 μM free C14:0, 1.24 mM NAD, 3.09 mM sodium lactate, and 1.31 mM $MgCl_2$ (pH 7.4), in a total volume of 7.5 mL. The digest was mixed at 500 rpm for 30 min at 37 °C. The microsomal supernatant (containing 94.5 mg of cytosolic protein) was trypsin-digested in one set of experiments and boiled for 30 min in another set. BSA was omitted from the incubation medium, because its effect was detrimental to the desaturase activity. [b] Means with no common letter are significantly different ($p < 0.05$).

The effect of trypsin digestion on the microsomal supernatant proteins is shown also in **Table 3**. It was found that proteolysis of FABP and other cytosolic proteins in the microsomal supernatant resulted in complete loss of the desaturase activity (**Table 3**). The control experiment was performed with untreated microsomal supernatant. These results suggest the presence of a protein factor in the microsomal supernatant that activates the direct desaturation of myristic acid. The protein factor is rather specific as it cannot be replaced by BSA. The question to be considered now is whether this protein factor binds the free myristic acid. If so, then the substrate for the direct desaturation by the hen microsomal Δ^9-desaturase would be in the form of a myristic acid−binding protein complex.

Effect of α-Bromohexadecanoate. It has been established that α-bromohexadecanoate competes with free fatty acids for the same binding site of fatty acid-binding proteins (*25*). The effect of α-bromohexadecanoate on the direct desaturation of myristic acid by hen hepatic microsomal Δ^9-desaturase was examined using different mole ratios of α-bromohexadecanoate/myristic acid (**Figure 5**). The linear decrease in specific activity observed with free myristic acid as a substrate in the presence of various concentrations of α-bromohexadecanoate probably reflects the reduction of myristic acid binding to FABP rather than the inhibition of the desaturation reaction per se. The desaturation of myritoyl-CoA was not affected by the presence of α-bromohexadecanoate. These results suggest that the specific protein factor in the microsomal supernatant is a FABP because it binds free fatty acid and the binding was completely inhibited by α-bromohexadecanoate. These results also suggest that the substrate for the direct desaturation by hen microsomal Δ^9-desaturase is in the form of a myristic acid−FABP complex. The decrease in the desaturase specific activity was directly related with the decrease in concentration of the myristic acid−FABP complex. Previous studies reported that hepatic fatty acid binding protein (hFABP) may specifically direct the utilization of fatty acids toward either esterification (*26*, *27*) or oxidative paths (*28*). Albumin was an ineffective replacer of hFABP in stimulating the activity of some microsomal enzymes utilizing fatty acids (*25*, *26*). This suggested the role of FABP in providing fatty acids to their enzymes may be more than a substrate solubilizer. **Table 2** reveals that FABP cannot be replaced by BSA due to the tight binding of the BSA−C14:0 complex (multiple binding sites) (*20*−*22*), making the C14:0

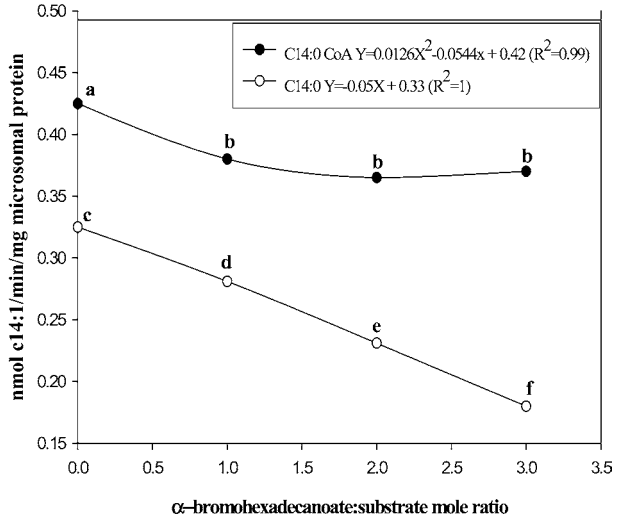

Figure 5. Effect of α-bromohexadecanoate/substrate mole ratios on the direct desaturation of myristic acid by the Δ^9-desaturase system of hen hepatic microsomes. The complete system contained 8 mg of microsomal protein, 94.5 mg of cytosolic protein, 40 mM potassium phosphate (pH 7.4), 120 mM sucrose, 133 μM free C14:0, α-bromohexadecanoate, 1.24 mM NAD, 3.09 mM sodium lactate, and 1.31 mM $MgCl_2$ (pH 7.4), in a total volume of 7.5 mL. The digest was mixed at 500 rpm for 30 min at 37 °C. When myristoyl-CoA was the substrate, 133 μM myristoyl-CoA replaced free C14:0. Means with no common letter are significantly different ($p < 0.05$).

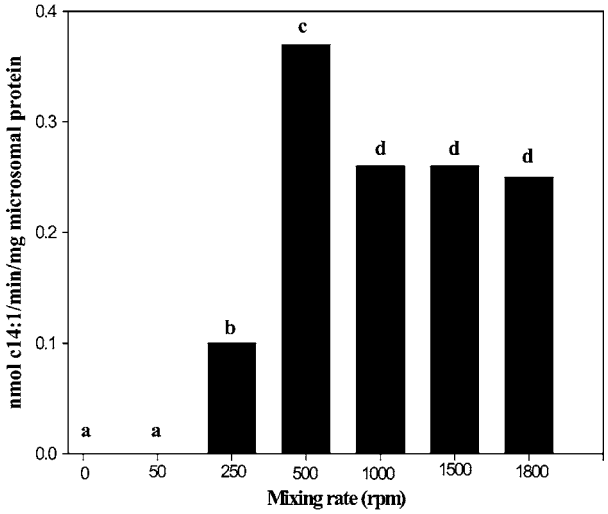

Figure 6. Effect of mixing rate on the direct desaturation of myristic acid–FABP complexes by the Δ^9-desaturase system of hen hepatic microsomes. The complete system contained 8 mg of microsomal protein, 94.5 mg of cytosolic protein, 40 mM potassium phosphate (pH 7.4), 120 mM sucrose, 133 μM free C14:0, 1.24 mM NAD, 3.09 mM sodium lactate, and 1.31 mM $MgCl_2$ (pH 7.4), in a total volume of 7.5 mL. The digest was mixed at 37 °C for 30 min. Means with no common letter are significantly different ($p < 0.05$).

Table 4. Effect of Substrate Concentrations on the Direct Desaturation of Myristic Acid–FABP Complexes by the Δ^9-Desaturase System of Hen Liver Microsomes[a]

substrate	C14:1[b] (M) (nmol/min/mg of microsomal protein)
0	0a
133	0.27b

[a] The complete system contained 8 mg of microsomal protein, 94.5 mg of cytosolic protein, 40 mM potassium phosphate (pH 7.4), 120 mM sucrose, 1.24 mM NAD, 3.09 mM sodium lactate, and 1.31 mM $MgCl_2$ (pH 7.4), in a total volume of 7.5 mL. The digest was mixed at 500 rpm for 30 min at 37 °C. [b] Means with no common letter are significantly different ($p < 0.05$).

inaccessible the active site of the Δ^9-desaturase. The FABP−fatty acid complex has one binding site (*29*), thus allowing easier access of C14:0 to the enzyme active site.

Oleic acid binds hFABP through the carboxyl group using ^{13}C NMR spectroscopy (*22*). The incubation of hFABP with labeled oleate reached an apparent equilibrium at a fatty acid to protein mole ratio of about 1 (*13*). Moreover, Brecher et al. (*13*) reported that the methyl esterification of the carboxyl group of long-chain fatty acids inhibits binding of the fatty acids to FABP. These findings lead to the conclusion that the FABP may regulate the accessibility of fatty acids in the desaturation reaction to the active site of the desaturase rather than just protect the membrane-bound desaturase from the cytotoxic effect of free fatty acids.

Effect of Mixing Rate. The effect of mixing rate on the desaturation of the myristic acid−FABP complex by hen hepatic microsomal Δ^9-desaturase was examined using mixing speeds from 0 to 1800 rpm. No desaturation was detected in the absence of mixing despite the presence of the microsomal supernatant containing FABP (**Figure 6**). This observation agrees with previously reported research (*2*, *7*, *8*, *11*, *12*, *14*−*16*) where no evidence was found for the direct desaturation of free fatty acids without being activated to the CoA derivatives. Production of myristoleic acid was observed at mixing speeds of >250 rpm. The optimal mixing rate for maximum product formation was 500 rpm (**Figure 6**). The decline in myristoleic acid production at mixing speeds of >500 rpm could be due to the rupturing of the microsomal membrane.

The possibility that free myristic acid was activated to its CoA derivative prior to the desaturation reaction in the presence of endogenous thiokinase, CoA, and ATP in the microsomal supernatant is unlikely because no desaturation was observed at mixing rates of <250 rpm despite the presence of the microsomal supernatant, as it is known that the activation reaction of free fatty acids does not require high shear mixing of the incubation medium. Moreover, the formation of myristoleic acid was not due to the potential presence of endogenous myristoyl-CoA, because in the absence of free myristic acid (substrate) no desaturation was observed (**Table 4**).

Different mixing rates were used to facilitate the transfer of the myristic acid−FABP complex (substrate) to the active site of the desaturase by direct collision to form productive enzyme−substrate complexes. The rate of transfer of the free fatty acid from hFABP to egg phosphotidylcholine membranes is influenced by both the structure of the free fatty acid ligand and the properties of the aqueous phase (pH, ionic strength, temperature) through which the free fatty acid must travel (*30*). Working with purified stearyl-CoA desaturase and acyl-CoA derivatives as substrates, free CoA and free fatty acids did not bind to the Δ^9-desaturase enzyme and did not inhibit the desaturation reaction (*31*). The secondary alcohol dehydrogenase provides an NADPH-dependent route to ethanol from acetyl-CoA and acetaldehyde (*32*).

The data presented here lead to the conclusion that when free myristic acid is bound to FABP in the form of protein−monomer complexes, its activation to the CoA derivative is not necessary in order to be desaturated by Δ^9-desaturase when a mixing rate of >250 rpm is used. Myristic acid−FABP

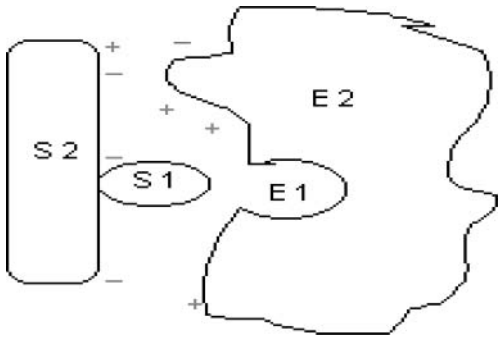

Figure 7. Enzyme–substrate complex illustrating both the geometrical and the physical complementarity between active site (E1) and reaction side (S1): E1 is the active site of the enzyme, E2 is the no-catalytic side of the enzyme, S1 is the reaction side of the substrate, and S2 is the guiding side of the substrate.

complexes probably serve as substrates for the Δ^9-desaturase at high mixing rates.

Substrate Specificity Studies. The variation in molecular structure between fatty acyl CoA and the free fatty acid gives rise to variation in reactivity. The only difference between the two molecules is the CoA esterified to the acyl group. This observation might be explained by the guiding and reaction side proposed hypothesis:

"*The formation of productive enzyme−substrate complexes occurs if S1 is complementary in shape, size, and chemical nature to E1, and S2 when needed, is properly guiding S1 to E1 without any steric hindrance.*"

Let us consider S = S1 + S2 and E = E1 + E2 (**Figure 7**), where S is the substrate; S1 is the reaction side, which determines the type of products; S2 is the guiding side, which determines the reactivity; E is the enzyme; E1 is the active site, which determines the type of reaction; and E2 is the noncatalytic side, which maintains the enzyme in its tertiary structure.

The guiding side (S2) affects reactivity in two general ways: (1) by the effect of long-range forces between S2 and E2 (mainly electrostatic and ion−dipole interaction) that facilitate the trafficking of the reaction side (S1) toward the active site (E1) and that activate the short-range forces (London−van der Waals dispersion) between S1 and E1 to induce a precise fit and (2) by its steric effect, which prohibits the formation of active site−reaction side (E1−S1) complexes, which lead to the formation of product. The steric effect of S2 is dependent on the configuration of E2. E2 is not a dead weight; it plays an important role in the catalytic activity of E.

We investigated several scenarios to verify the validity of this hypothesis.

First Scenario: Fatty Acyl CoA Derivative and Δ^9-Desaturase.

S: C14:0CoA (substrate)	E: Δ^9-desaturase
S1: C14:0 (reaction side)	E1: active site
S2: CoA (guiding side)	E2: noncatalytic side

The transfer of C14:0 (S1) to the catalytic domain of the Δ^9-desaturase (E1) was accomplished via direct collision interaction through the electrostatic attractions between the water-soluble CoA moiety (S2) and the hydrophilic part (E2) of the membrane-bound Δ^9-desaturase, which exposes the catalytic domain to the aqueous environment. Positively charged arginyl residues of the stearyl-CoA desaturase may be involved in the binding of the CoA moiety of the negatively charged substrate, as the substrate (stearyl-CoA) has three phosphate groups and bears three to four negative charges (*31*).

The guiding side CoA (S2) facilitates the trafficking of the hydrophobic myristic acid (S1) to traverse the aqueous phase to reach the membrane-bound Δ^9-desaturase. There was no steric effect observed with CoA. Myristoyl-CoA did meet the conditions of the proposed concept. The Δ^9-desaturase specific activity expressed as nanomoles of C14:1 per minute per milligram of microsomal protein was 0.50. Liposome-bound stearyl-CoA is the true substrate for the purified Δ^9-desaturase in the absence of BSA (*6*). Bound stearyl-CoA undergoes lateral diffusion in the bilayer to reach the active site of the enzyme. However, in the presence of BSA, the possibility that myristoyl-CoA is reaching the enzyme by lateral diffusion is unlikely due to the ability of the albumin to remove the fatty acyl-CoA from the liposomes. The transfer of substrate by direct collision is more likely.

The addition of albumin to unilamellar vesicles at a molar ratio equivalent to that of oleoyl CoA effectively removed oleoyl CoA from the vesicle and resulted in an oleoyl CoA−albumin complex (*32*). Albumin is not necessary for the desaturation reaction per se. The role of albumin is to protect the acyl-CoA derivatives from the acyl transferase present in the cytosol (*27*).

Second Scenario: Free Fatty Acids and Δ^9-Desaturase.

S: C14:0	E: Δ^9-desaturase system
S1: C14:0 (reaction side)	E1: active site
S2: absent (guiding side)	E2: noncatalytic side

Myristic acid as a free monomer did not meet the conditions of the hypothesis. The desaturation reaction did not occur because the guiding side (S2) was absent. The reaction is substrate-transfer limited, instead of being limited to the kinetics of the reaction. However, when the microsomal supernatant containing FABP was added to the incubation medium coupled with a mixing speed of 500 rpm, the specific activity observed was 0.268 nmol/min/mg of microsomal protein. In this system, neither CoA nor ATP was added. Free myristic acid binds to FABP to form a myristic acid−FABP complex, which ultimately dissociates in the aqueous phase. When exposed to protein (BSA or FABP), 50% of the fatty acids contained within liposomes could become protein-bound, and the newly formed fatty acid−protein complex dissociates from the bilayer surface to the aqueous space (*32*). They also reported that the movement of fatty acid from protein to liposomes is a much slower process.

The possibility that myristic acid reaches the active site of the Δ^9-desaturase by lateral diffusion in the bilayer is unlikely to be due to the effect of binding proteins. The transfer of myristic acid to the Δ^9-desaturase catalytic domain was accomplished via random collision between the myristic acid−FABP complexes and the membrane-bound Δ^9-desaturase. The random collisions were generated using different mixing rates. A mixing rate of 500 rpm was necessary to overcome diffusion obstacles due to the properties of the aqueous phase (pH, ionic strength, temperature), through which the myristic acid−FABP complexes have to travel. The desaturation reaction did not occur in the absence of FABP despite the use of different mixing rates. These results suggest that FABPs play a key role in the formation of enzyme−substrate complexes, probably via a physical effect on the myristate.

The collision between myristic acid−FABP complex and the membrane-bound Δ^9-desaturase activates the short-range forces between the myristic acid (S1) and the active site (E1) of the desaturase, therefore inducing a precise fit. The short-range forces are London−van der Waals dispersions and weak polar attraction due to the polarizable π electrons of the carbonyl group. In this case, the FABP is the guiding side (S2) and

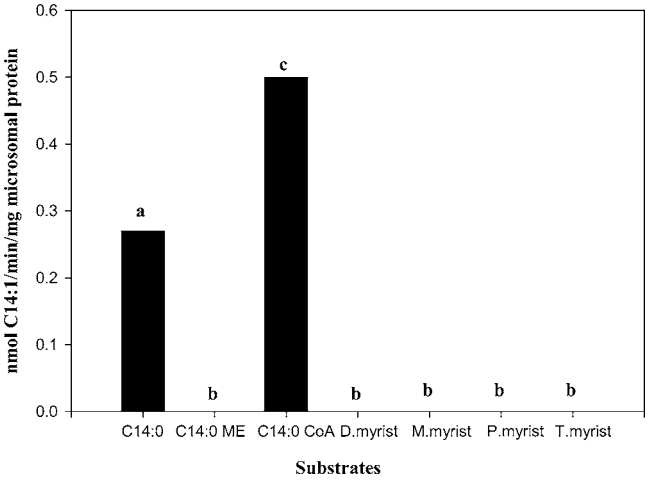

Figure 8. Effect of different substrates on the specific activity of the hen microsomal Δ^9-desaturase system. The complete system contained 8 mg of microsomal protein, 94.5 mg of cytosolic protein, 40 mM potassium phosphate (pH 7.4), 120 mM sucrose, 1.24 mM NAD, 3.09 mM sodium lactate, and 1.31 mM $MgCl_2$ (pH 7.4), in a total volume of 7.5 mL. The digest was mixed at 500 rpm for 30 min at 37°C. Means with no common letter are significantly different ($p < 0.05$).

myristic acid is the reaction side (S1). This observed effect was specific for FABP and could not be duplicated by albumin (**Table 2**), giving more weight to the argument that the effect of FABP is specific in regulating the desaturase activity.

The reactivity of the reaction is determined by the nature of the guiding side (S2). Under the experimental conditions used, the guiding side CoA was more reactive than the guiding side FABP because the specific activity of the desaturase was higher (**Figure 8**).

Third Scenario: Trimyristin and Δ^9-Desaturase System.

S: trimyristin	E: Δ^9-desaturase
S1: C14:0 (reaction side)	E1: active site
S2: glycerol backbone (guiding side)	E2: noncatalytic side

The desaturation reaction did not occur even in the presence of FABP and the use of different mixing rates to overcome diffusion obstacles due to the properties of the aqueous phase (pH, ionic strength, temperature) through which the substrate must travel. This lack of desaturation might be due to the steric effect exerted by the glycerol backbone (S2) on E2. Trimyristin did not meet the conditions of the proposed hypothesis, and thus no desaturation was observed. The same effect was observed with 1-monomyristoyl-*rac*-glycerol (C14:0), 1,2-dimyristoyl-*rac*-glycerol (C14:0), myristic acid methyl ester, and L-α-phosphotidylcholine dimyristoyl (C14:0) (**Figure 8**).

Conclusions. The current research effort was directed at understanding the regulation of the Δ^9-desaturase system of hen liver microsomes using myristoyl-CoA and myristic acid−FABP complexes as substrates. Experimental evidence has been presented to show that the variation in molecular structure between myristoyl-CoA and myristic acid−FABP complexes gives rise to variation in reactivity. CoA and FABP are guiding molecules. There are positive influences pulling reactants, but negative repulsion plays a major role in the way reactants are guided. Attraction and repulsion can act over short and long ranges. Short-range interaction requires contacts with membrane-bound Δ^9-desaturase, and long-range interactions occur by diffusible factors that set up gradients. Unraveling the mechanism of these interactions is complicated by the fact that guidance molecules can have bifunctional roles. The same molecule can be attractive or repulsive, short- or long-range.

The frustrations of designing enzyme inhibitors are nothing new. This study has shed some light on the effect of the substrate guiding side (S2) on the reactivity of enzymatic reactions. Every enzyme has an inhibitor, and maybe with a better understanding of the role of the substrate guiding side in enzymatic reactions, researchers will be able to find a more efficient way to design enzyme inhibitors.

As the Δ^9-desaturase is inactive on triacylglycerol substrates, the application of the desaturation process on edible fat rich in myristic acid, for example, milk fat, necessitates a lipase-catalyzed hydrolysis of milk fat triacylglycerols to liberate the free fatty acids (FFAs) prior to the desaturation reaction and finally to re-esterify the FFAs.

LITERATURE CITED

(1) Brett, D.; Morris, L. J.; James, A. T. Specificity of the fatty acid desaturases. The conversion of saturated to monoenoic acids. *Arch. Biochem. Biophys.* **1971**, *143*, 535−547.

(2) Masoro, E. J. Biosynthesis of fatty acids. In *Physiological Chemistry of Lipid in Mammals*; Masoro, E. J., Ed.; Saunders: Philadelphia, PA, 1968; Chapter 4, pp 58−73.

(3) Holloway, P. W. A requirement for the three protein components in microsomal stearyl coenzyme A desaturation. *Biochemistry* **1971**, *10*, 1556−1560.

(4) Spartz, L.; Strittmatter, P. A form of reduced nicotinamide adenine dinucleotide cytochrome b_5 reductase containing both the catalytic site and an additional hydrophobic membrane-binding segment. *J. Biol. Chem.* **1973**, *248*, 793−799.

(5) Strittmatter, P.; Spartz, L.; Corcoran, D.; Rogers, M. J.; Setlow, B.; Reline, R. Purification and properties of rat liver microsomal stearoyl coenzyme A desaturase. *Proc. Natl. Acad. Sci. U.S.A.* **1974**, *71*, 4565−4569.

(6) Enoch, H. G.; Catala, A.; Strittmatter, P. Mechanism of rat liver microsomal stearyl-CoA desaturase. *J. Biol. Chem.* **1976**, *251*, 5095−5103.

(7) Raju, P. K.; Reiser, R. Stimulation of stearoyl CoA desaturase activity by sn-glycerol-3-phosphate in mouse liver microsomes. *Biochim. Biophys. Acta* **1972**, *280*, 267−274.

(8) Brenner, R. R. The desaturation step in the animal biosythesis of polyunsaturated fatty acids. *Lipids* **1971**, *6*, 567−575.

(9) Gurr, M. I.; Robinson, M. P.; James, A. T. The mechanism of formation of polyunsaturated fatty acids by photosynthetic tissue. The tight coupling of oleate desaturation with phospholipid synthesis in *Chlorella vulgaris*. *Eur. J. Biochem.* **1969**, *9*, 70−78.

(10) Pugh, E. L.; Kates, M. Direct desaturation of eicosatrienoyl lecithin to arachidonoyl lecithin by rat liver microsomes. *J. Biol. Chem.* **1977**, *252*, 68−73.

(11) Cook, H. W.; Spence, M. W. Formation of monoenoic fatty acids by desaturation in rat brain homogenate. *J. Biol. Chem.* **1973**, *248*, 1786−1793.

(12) Pugh, E. L.; Kates, M. Desaturation of phosphatidylcholine and phosphatidylethanolamine by a microsomal enzyme system from *Candida lipotica*. *Biochim. Biophys. Acta* **1969**, *316*, 305−316.

(13) Brecher, P.; Saouaf, R.; Sugarman, J. M.; Eisenberg, D.; Larosay, K. Fatty acid transfer between multilamellar liposomes and fatty acid binding proteins. *J. Biol. Chem.* **1984**, *259*, 13395−13401.

(14) Catala, A.; Nervi, A. M.; Brenner, R. R. Separation of a protein factor necessary for the oxidative desaturation of fatty acids in the rat. *J. Biol. Chem.* **1975**, *250*, 7481−7484.

(15) Cook, H. W. In vitro formation of polyunsaturated fatty acids by desaturation in rat brain: some properties of the enzymes in developing brain and comparison with liver. *J. Neurochem.* **1978**, *30*, 1327−1334.

(16) Voet, D.; Voet, J. G. Lipid metabolism. In *Biochemistry*; Wiley: New York, 1990; Chapter 23, pp 618−665.

(17) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265−275.
(18) Morrison, W. R.; Smith, L. M. Preparation of fatty acid methyl esters and dimethacetals from lipids with boron trifluoride−methanol. *J. Lipid Res*. **1964**, *5*, 600−608.
(19) Sackmann, E.; Träuble, H. Studies on the crystalline-liquid crystalline phase transition of liquid model membranes. *J. Am. Chem. Soc*. **1971**, *97*, 4492−4498.
(20) Parks, J. S.; Cistola, D. P.; Small, D. M.; Hamilton, J. A. Interactions of the carboxyl group of oleic acid with bovine serum albumin: A ^{13}C NMR study. *J. Biol. Chem*. **1983**, *258*, 9262−9269.
(21) Hamilton, J. A.; Cistola, D. P.; Morrisett, J. D.; Sparrow, J. T. Interactions of myristic acid with bovine serum albumin: A ^{13}C NMR study. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 3718−3722.
(22) Cistola, D. P.; Small, D. M.; Hamilton, J. A. Carbon 13 NMR studies of saturated fatty acids bound to bovine serum albumin. *J. Biol. Chem*. **1987**, *262*, 10971−10979.
(23) Collins, D. M.; Hargis, P. S. Distribution of fatty acid binding proteins in tissues and plasma of *Gallus domesticus*. *Comp. Biochem. Physiol*. **1989**, *92*, 283−289.
(24) Ockner, R. K.; Manning, J. A. Fatty acid binding protein. *J. Clin. Invest*. **1976**, *58*, 632−641.
(25) Wu-Rideout, M. Y. C.; Elson, C.; Shargo, E. The role of fatty acid binding protein on the metabolism of fatty acids in isolated rat hematocytes. *Biochem. Biophys. Res. Commun*. **1976**, *71*, 809−816.
(26) Iritani, N.; Fukuda, E.; Inogushi, K. A possible role of Z protein in dietary control of hepatic triacylglycerol synthesis. *J. Nutr. Sci. Vitaminol*. **1980**, *26*, 271−277.
(27) AppleKvist, E. L.; Dallner, G. Possible involvement of fatty acid binding protein in peroxisomal β-oxidation of fatty acids. *Biochim. Biophys. Acta* **1980**, *617*, 156−160.
(28) Glatz, J. F. C.; Veerkamp, J. H. A radiochemical procedure for the assay of fatty acid binding by proteins. *Anal. Biochem*. **1983**, *132*, 89−95.
(29) Kim, H. K.; Storch, J. Free fatty acid transfer from rat liver fatty acid-binding protein to phospholipid vesicles. *J. Biol. Chem*. **1992**, *267*, 77−82.
(30) Enoch, H. G.; Strittmatter, P. Role of tyrosyl and arginyl residues in rat liver microsomal stearyl coenzyme A desaturase. *Biochemistry* **1978**, *57*, 4927−4932.
(31) Brudett, D.; Zeikus, J. G. Purification of acetaldehyde dehydrogenase and alcohol dehydrogenases from *Thermoanaerobacter ethanolicus* 39E and characterization of the secondary-alcohol dehydrogenase as a bifunctional alcohol dehydrogenase-acetyl CoA reductive thioesterase. *J. Biochem*. **1994**, *302*, 163−170.
(32) Lichtenstein, A. H.; Small, D. M.; Brecher, P. Interaction of oleoyl coenzyme A with phospholipid bilayers. *Biochemistry* **1982**, *21*, 2233−2241.

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