Separation of a Protein Factor Necessary for the Oxidative Desaturation of Fatty Acids in the Rat*

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Microsomes from rat liver were extracted by low ionic strength solutions. Extracted microsomes lost most of the linoleic acid desaturation activity. The addition of the extract back into the extracted microsomes was necessary to restore full desaturation activity. The soluble fraction had no desaturation activity. The existence of a soluble factor loosely bound to the microsomes, stable to sonication, and unstable to heat and trypsin digestion was recognized. This protein could not be replaced by albumin. The factor was also essential for the oxidative desaturation of palmitic, stearic, linoleic, and γ-linolenic acid. The present experiment suggests that the protein factor is not NADH-cytochrome b₅ reductase, cytochrome b₅, or the cyanide-sensitive factor.

Several attempts have been made in different laboratories to separate and purify stearoyl coenzyme A desaturase and other fatty acid desaturases of microsomes, and to determine the enzymatic components necessary for the reaction. Jones et al. (1) showed that stearoyl-CoA desaturation reaction required the presence of microsomal lipids. In 1968, Gurr et al. (2) partially "solubilized" a stearoyl-CoA desaturase using 1 M phosphate buffer. Shortly afterward, Gurr and Robinson (3) partially separated and purified the enzyme using non-ionic detergents. In 1971, Oshino et al. (4) demonstrated the function of a microsomal cytochrome b₅ in fatty acid desaturation, and Gaylor et al. (5) isolated a cyanide-binding protein. Holloway and Wakil (6) also demonstrated the requirement of reduced nicotinamide adenine dinucleotide-cytochrome b₅ reductase in stearoyl-CoA desaturation. In 1971, Holloway (7) extended the research, and showed the requirement for three protein components. In 1972, Shimakata et al. (8) confirmed these results and solubilized the cytochrome b₅-sensitive factor. On the basis of these investigations, all workers assumed that the multienzymatic system was limited only to the aforementioned microsomal proteins and phospholipids. Nevertheless, in 1972 (9), the authors were able to separate from the microsome a "soluble" fraction that was necessary for the full activity of linoleic acid desaturation to γ-linolenic acid. Further information on the constitution and properties of this soluble factor is here reported.

EXPERIMENTAL PROCEDURE

Materials—[1-¹⁴C]Linoleic acid (57 mCi/mm), [1-¹⁴C]palmitic acid (57 mCi/mm), α-[1-¹⁴C]linolenic acid (58 mCi/mm), and [1-¹⁴C]stearic acid (54 mCi/mm), were provided by The Radiochemical Centre, Amersham, England, and were 99% pure. Trypsin, type I, and trypsin inhibitor were purchased from Sigma Chemical Co., St. Louis, Mo. Linoleyl-CoA and labeled linoleyl-CoA were prepared by the procedure of Kornberg and Price (10). [1-¹⁴C]Linoleic acid (57 mCi/mm), α-[1-¹⁴C]linolenic acid (58 mCi/mm), and [1-¹⁴C]stearic acid (54 mCi/mm), were provided by The Radiochemical Centre, Amersham, England, and were 99% pure. Trypsin, type I, and trypsin inhibitor were purchased from Sigma Chemical Co., St. Louis, Mo. Linoleyl-CoA and labeled linoleyl-CoA were prepared by the procedure of Kornberg and Price (10). The present experiment suggests that the protein factor is not NADH-cytochrome b₅ reductase, cytochrome b₅, or the cyanide-sensitive factor.

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Using linoleyl-CoA as substrate, 1 mg of microsomal protein and 8 
mmol of "C-labeled acyl-CoA were incubated for 20 min at 35° with 3 
ml of the same cofactors solution as above, but without CoA. The final 
volume was also 3.2 ml.

Both reactions were stopped by addition of 2 ml of 10% methanolic 
KOH. Fatty acids were saponified and acidified, and extracted three with 2 ml of petroleum ether (30-40° b.p.). They were esterified 30 min with 3 N HCl in methanol. Conversion was measured by gas-liquid radiochromatography in a Pye apparatus with proportional counter (13).

NADH-Cytochrome b5 Reductase—NADH-cytochrome b5 reductase was assayed at 25° by measuring the NADH-ferricyanide reductase activity of the enzyme according to Strittmatter (14). The reaction mixture contained 0.025 μmol of reduced pyridine nucleotide and 0.05 μmol of potassium ferricyanide in a final volume of 0.25 ml of 0.1 M Tris-acetate buffer (pH 8.1) containing 1.0 mM EDTA. The decrease in absorbance at 340 nm was followed as a function of time.

Other Assays—To each 6 ml of Sp containing 0.3 mg/ml of protein, a solution of 6 mg of trypsin in 0.25 ml of phosphate buffer (pH 7.4) was added, and the mixture was incubated for 1 hour at 37°. Proteolysis was stopped with the addition of 12 mg of trypsin inhibitor. Me (10 mg) suspended in 0.2 ml of homogenizing solution was incubated similarly with 10 mg of trypsin; 20 mg of trypsin inhibitor were added.

The effect of sonication on M, Me, and Sp was also investigated. Sonication at 100 watts was performed at 0-4° under nitrogen in a Biosonik II apparatus for three periods of 1-min each.

Liposomes were prepared according to Rogers and Strittmatter (15) with lipids extracted by the Folch method (16) from Me or Sp.

The lipid composition of Sp was investigated. Sp separated at 0-4° was concentrated by lyophilization. An aliquot of the concentrated lipids was incubated with 10 nmol of labeled linoleic acid and necessary cofactors for 20 min at 35°. (For other details, see under “Experimental Procedures.”)

**RESULTS**

**Extraction of Sp**—Results in Table I show that after 15 min of anaerobic preincubation of liver microsomes at 35° in a solution containing 0.15 M KCl, 0.25 M sucrose, 0.04 M phosphate buffer, 0.04 M NaF, and 1.5 mM glutathione, a “solubilized” microsomal component may be separated by ultracentrifugation at 100,000 x g for 1 hour. The capacity of the extracted microsomes (Me) to desaturate linoleic acid was significantly reduced, whereas the supernatant fraction (Sp) had no measurable desaturase activity. However, the readdition of Sp to Me restored the activity nearly to the original value. These results confirmed previous work, and demonstrated the separation of a factor, or factors, bound to the microsomes. This factor was necessary for the full desaturase activity of the microsomes. Furthermore, the separation of the soluble factor was also achieved by gentle shaking of the microsomes in the same solution for a few min in the cold. This mild procedure has the advantage of producing less inactivation of the desaturase, and it has been used hereafter as a standard method of extraction.

**Sonication**—Sonication under conditions described under “Experimental Procedure” evoked a decrease of the relative desaturation activity of the microsomes (M) from 100% to 56.4%. However, sonication of Me did not decrease their remaining desaturation activity (53.2%), but reduced their capacity to restore full desaturation activity when the system was reconstituted by addition of nonsonicated Sp. When sonicated Sp was added to normal Me, the relative desaturation capacity of the complete system was restored to 53.2% to 103.2%. Therefore, this experiment shows that sonication affects the microsomes but not the Sp. It could mean that the particulate component of the system is the only one altered by sonication.

**Effect of Sp Concentration on the Reactivation of Me**—A study of the effect of the amount of Sp necessary to reactivate Me is shown in Fig. 1. The reactivation of the desaturation reaction is dependent on the amount of Sp added, and the saturation curve reaches a plateau when the amount of Sp added is approaching the amount extracted from the microsomes. Since it was also found that the addition of Sp to M did not increase the desaturation activity (9), it is possible to consider that the original microsomes (M) were already saturated with the activating factor or factors extracted by centrifugation.

**Behavior of Me and Sp on the Desaturation of Different Substrates**—The activating effect of Sp on the Me was not specific for linoleic acid desaturation to γ-linolenic acid (Fig. 2). It was also shown on the desaturation of stearic to oleic acid, palmitic to palmitoleic acid, and γ-linolenic to octadec-6,9,12,15-tetraenoic acid. Therefore, the desaturation-reactivation phenomenon is evoked in both 6 and 9 desaturations. Be-

**TABLE I**

Effect of temperature on extraction of microsomal “soluble factor” involved in oxidative desaturation of linoleic acid to γ-linolenic acid

<table>
<thead>
<tr>
<th>Fractions</th>
<th>35° relative desaturation</th>
<th>0-4° relative desaturation</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Me</td>
<td>17.9</td>
<td>23.8°</td>
</tr>
<tr>
<td>Sp</td>
<td>Not measurable</td>
<td>Not measurable</td>
</tr>
<tr>
<td>Me + Sp</td>
<td>71.9</td>
<td>98.7°</td>
</tr>
</tbody>
</table>

*M, whole microsomes; Me, extracted microsomes; Sp, supernatant containing the “soluble faction.”

* A second extraction of Me by the same procedure reduced the remaining desaturation activity from 29.6 to 6.7.

Fig. 1. Effect of Sp concentration on the restoration of linoleate desaturation activity of extracted microsomes. Me and Sp were separated at 35° under conditions described under “Experimental Procedure.” Desaturation activity was measured by incubation of 5 mg of Me and increasing amounts of Sp, with 20 nmol of labeled linoleic acid and cofactors. One hundred per cent value was the desaturation activity of 5 mg of Me protein and 1.1 mg of Sp that correspond to the proportion present in M. See others under “Experimental Procedure.”
sides, it was necessary to discard the possibility that the reactivation of the desaturation reaction was not evoked through the acyl-CoA-synthetizing reaction. For this reason, the deactivation-reactivation phenomenon was also tested using [1-14C]linoleyl-CoA as substrate. The results are shown in Table II, and prove that the effect is independent of linoleyl-CoA synthesis.

**Effect of Heat on the Capacity of Sp to Restore the Desaturation Activity of Me**—The effect of heat on the capacity of Sp to restore the desaturation capacity of Sp is shown in Table III. Sp obtained in the usual way at 0-4° was heat-unstable when heated for 10 min at 60° and 5 min at 70° under nitrogen, for it lost most of its activating effect on the desaturation reaction. However, we may point out that in previous experiments, little deactivation of Sp was found when Sp was heated at 70° for 5 min (9, 17). These apparently contradictory results cannot be explained for the moment. The activity of Sp was completely lost after 15 min of boiling. At the same time, boiling produced precipitation of proteins.

**Effect of Trypsin on Sp and Me**—The effect of trypsin digestion on Sp is shown in Table IV. It was found that proteolysis inhibited Sp capacity to reactivitate Me. A control experiment performed by incubation of Sp with trypsin inhibited with trypsin inhibitor did not modify Sp capacity to reactivate the desaturase reaction. Therefore, full activity of Sp was dependent on an unchanged protein structure. As is clearly shown in Table IV, an intact protein structure was also necessary in Me.

The protein factor of Sp is rather specific, since it cannot be replaced by albumin, as is also shown in the same table.

**Delipidization of Sp**—Lipids are normally present in Sp. They were extracted by the procedure of Folch et al. (16). The presence of phosphatidylcholine, phosphatidylethanolamine, free fatty acids, and neutral lipids was recognized by thin layer chromatography in Silica Gel G. It is important to determine to what extent these lipids are necessary to maintain full Sp activity. Previous results have shown that acetone extraction of Sp diminished the capacity of Sp to restore linoleic acid desaturation activity of Me (17). However, the reconstitution of the system with liposomes was not successful.

In a new experiment we removed the lipids from Sp with ether, but this treatment did not reduce the capacity of Sp to restore the desaturation activity of the system (Table IV). The addition of liposomes to the mixture of lipid-extracted Sp and Me did not increase significantly the desaturation activity of the system. Therefore, it may be deduced that apparently, the lipids extracted by ether are not necessary components of Sp-activating factor. Nevertheless, ether did not remove exhaustively the lipids from Sp, since additional amounts of

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Relative desaturation activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>100.0</td>
</tr>
<tr>
<td>Me</td>
<td>18.0</td>
</tr>
<tr>
<td>Me + Sp</td>
<td>75.7</td>
</tr>
<tr>
<td>Me + Sp (trypsin-treated)</td>
<td>38.7</td>
</tr>
<tr>
<td>Me (trypsin-treated)</td>
<td>1.8</td>
</tr>
<tr>
<td>Me + [Sp + (trypsin + trypsin inhibitor)]</td>
<td>77.5</td>
</tr>
<tr>
<td>Me + 0.5 mg of albumin</td>
<td>19.3</td>
</tr>
<tr>
<td>Me + delipidized Sp</td>
<td>81.1</td>
</tr>
<tr>
<td>Me + delipidized Sp + liposomes</td>
<td>86.5</td>
</tr>
</tbody>
</table>

*Sp was treated with trypsin previously inhibited with trypsin inhibitor.*
them could be extracted from Sp with Cl₆CH/CH₃OH (2/1, v/v). However, this solvent mixture denatured the proteins of Sp, and no enzymatic activity was then measurable. It is possible that lipids remaining in Sp after ether extraction may still play a role in the capacity of Sp to restore the desaturation activity of Me. However, it was found that liposomes prepared with lipids extracted from whole microsomes or from Sp with Cl₆CH/CH₃OH (2/1, v/v) were unable to increase the fatty acid desaturation activity of Me.

NADH-Cytochrome b₅ Reductase—In order to investigate to what extent the preparation of Sp could involve the extraction of proteins firmly bound to the microsomal membrane, the activity of NADH-ferricyanide reductase was measured in Me and Sp. As expected, the relative activity of the reductase in Sp was very low compared to Me. It was less than 4.8% of Me activity. Evidently, the mild extraction procedure used to obtain Sp was inefficient to separate this integral protein out of microsomal membranes.

**DISCUSSION**

The desaturation reaction of polyunsaturated fatty acids is produced in the microsomes of rat liver on the acyl-CoA thioesters and requires NADH and O₂. Up until now, it has been possible to demonstrate that stearyl-CoA desaturation in mammals requires a flavoprotein (NADH-cytochrome b₅ reductase), cytochrome b₅, and a cyanide-sensitive factor. They are components of the microsomal electron transport chain. Besides, they are integral proteins of the endoplasmic reticulum, since to be removed from the microsomes, they required detergents or other drastic procedures that interact with the lipidic part of the membrane (6–8, 14, 18). As is shown in Table I, we have been able to separate a soluble factor out of the microsomes that is necessary to recover the full desaturation activity of the extracted microsomes. This factor is loosely bound to the microsomes since it is separated with solutions of low ionic strength. Therefore, using Singer’s nomenclature (19), we may consider that this factor is an extrinsic component. The same or similar factors are necessary for both 6 and 9 desaturations (Fig. 2). Besides, this factor is not involved in the acyl-CoA synthetase (Table II).

The reactivity of the factor is not modified by sonication, but is deactivated by boiling and trypsin digestion (Tables III and IV). Therefore, a protein structure is involved in the soluble factor activity. This protein is specific, since it cannot be replaced by albumin (Table IV).

Therefore, all of these results show that this factor is obviously neither cytochrome b₅, nor NADH-cytochrome b₅ reductase, nor the cyanide-sensitive factor, since all of these microsomal components are integral proteins of the membrane and require rather drastic treatments to be liberated from the microsomes (7, 8). Our extraction procedures were mild (Table I), as is shown by the low NADH-ferricyanide reductase activity found in Sp. We also consider that our protein factor is not the desaturase itself, since all experiments carried out until now have shown that detergents of high ionic strength solutions are necessary to remove this enzymatic activity from the microsomes (6–8).

Moreover, Oshino et al. (4) have suggested that the cyanide-sensitive factor could be the desaturase itself, and its activity is modified by the diet (20). However, we found that in spite of the induction of desaturase activity by dietary factors and hormones (17, 21, 22), the Sp activity is apparently not modified by these factors (23). How this soluble protein factor binds to the Me and how it functions in fatty acid desaturation are still unanswered questions.

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