Role of the Enoyl Reductase Domain in the Regulation of Fatty Acid Synthase Activity by Interdomain Interaction

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Fatty acid synthase from the uropygial gland of goose was inhibited by increasing the [NADP]/[NADPH] ratio. NADP inhibition of the overall activity of fatty acid synthase, the ketoreductase, and the enoyl reductase were competitive with respect to NADPH with $K_i$ values of 6 $\mu$M, 40 $\mu$M, and 280 $\mu$M, respectively. The other component activities of fatty acid synthase were not affected by NADP with one exception; the condensing activity was severely inhibited. This inhibition was noncompetitive with respect to malonyl-CoA and hexanoyl-CoA. The inhibition of condensing activity by NADP was abolished by selective modification of the NADPH binding site of the enoyl reductase domain with pyridoxal phosphate. That the binding of NADPH, which triggers dimerization of fatty acid synthase peptides, occurs at the enoyl reductase domain was shown by the inability of NADPH to dimerize the pyridoxal phosphate-modified enzyme although this enzyme could be dimerized by high ionic strength. Proteolysis of the native enzyme was inhibited by NADP but the proteolysis of the modified enzyme was not. These results strongly suggest that enoyl reductase domain of fatty acid synthase plays a key role in the interdomain interactions which regulate the activity of the enzyme via the [NADP]/[NADPH] ratio.

Fatty acid synthase from goose uropygial gland catalyzes the synthesis of n-fatty acids from malonyl-CoA and multiple methyl branched fatty acids from methylealmonoyl-CoA using acetyl-CoA as the preferred primer and NADPH as the specific reductant (1). This enzyme appears to be identical with that in goose liver, and all of the physical, chemical, and catalytic properties of the enzyme from the gland are quite similar to those of the enzyme from other vertebrate sources. It consists of two equal molecular weight peptides, each containing one 4'-phosphopantetheine (1), one active serine at the thioesterase domain (2, 3), one essential arginine at the ketoreductase domain (4), and one essential arginine and lysine each at the enoyl reductase domain (4, 5). Most, if not all, of these structural features are also shared by fatty acid synthases from other vertebrates (6-9). Although long term regulation of fatty acid synthase activity, which depends on the enzyme level, has been studied in several systems, little is known about the short term regulation of fatty acid synthase activity. Since each peptide appears to contain seven different domains which catalyze a sequential set of reactions, binding of regulatory ligands to one domain might regulate the activity of other domains. However, such aspects do not appear to have been systematically explored. The recent finding that NADPH binding specifically to the enoyl reductase site can be prevented by chemical modification (5, 10) allowed us to examine the role of this nucleotide binding site in the regulation of this enzyme activity. The results presented in this paper strongly suggest that the NADP/NADPH binding to the enoyl reductase domain plays a key role in the regulation of this enzyme activity.

EXPERIMENTAL PROCEDURES

Materials—NADP, NADPH, pyridoxal 5'-phosphate, clostripain (EC 3.4.22.8), and all substrates for enzyme assays were purchased from Sigma Chemical Co. NADH was from Alfa Inorganics, Inc. l-Tosylamido-2-phenylethyl chloromethyl ketone trypsin was obtained from Worthington Biochemical Corp. NaH$^3$CO$_3$ was purchased from New England Nuclear. [1-14C]Acetyl-CoA and [2-14C]Malonaldehyde-CoA were prepared as described before (11, 12).

Enzymes and Assays—Fatty acid synthase and pyridoxal phosphate-modified enzyme were prepared as described previously (5, 10). Overall fatty acid synthase activity and all the component activities were measured as indicated elsewhere (5) except the transacylases in which case acyl pantetheine was extracted by 1-butanol and the radioactivity in the 1-butanol extract was determined (13). Condensation activity was also measured spectrophotometrically by monitoring absorbance change at 290 nm (14). The specific activities of overall fatty acid synthase, condensation, ketoreductase, and enoyl reductase activities were 0.8, 0.03, 2.50, and 0.09 nmol/min/mg of protein, respectively.

Inhibition Studies—NADP was added to the enzyme before addition of substrates. Controls had the buffer without NADP. Other details are given in the text.

Dissociation and Association of the Enzyme—Fatty acid synthase and pyridoxal phosphate-modified enzyme (6-7 mg/ml) were dialyzed against either 1 mM sodium phosphate buffer containing 1 mM dithioerythritol and 1 mM EDTA (pH 8.0) or buffer containing 55 mM glycine, 0.44 mM Tris, 1 mM dithioerythritol, and 1 mM EDTA (pH 8.0) for 72 h. After dialysis either NADPH (0.2 mM) or KCl (0.2 M) were added and incubated at room temperature for 30 min before measuring the extent of association by ultracentrifugation and by enzyme activity. A Spinco model E ultracentrifuge equipped with schlieren optics was used in all sedimentation velocity experiments. Centrifugations were carried out at 20°C with a rotor speed of 56,000 rpm.

Proteolysis of the Enzyme—Fatty acid synthase was incubated at 30°C with clostripain in 100 mM sodium phosphate buffer, pH 7.0, containing 1 mM EDTA, 0.5 mM dithioerythritol, and 10% ethanol. At fixed time intervals aliquots were removed from the reaction mixture, diluted, and assayed immediately for overall fatty acid synthase activity. Controls contained all additions except clostripain. Proteolysis of fatty acid synthase and pyridoxal phosphate-modified enzyme were done at 30°C in 100 mM phosphate buffer, pH 7.0, containing 1 mM EDTA and 0.5 mM dithioerythritol. At various intervals aliquots were removed, diluted, and assayed immediately for

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Enoyl Reductase in Regulation of Fatty Acid Synthase Activity

Protein and Radioisotope Determination—Protein was measured by the method of Lowry et al. (15) using bovine serum albumin as a standard. All the radioactive samples were dissolved in Scintiverse (Fisher), and the radioactivity was determined by scintillation spectrometry using a Packard Tri-Carb 460 CD liquid scintillation spectrometer with attached programmable disintegrations per min converter.

RESULTS

Effect of NADP-NADPH Ratio on the Component Activities and Overall Activity of Fatty Acid Synthase—To test whether fatty acid synthase activity is affected by the oxidation-reduction balance reflected in the [NADP]:[NADPH] ratio, the effect of NADP, at constant but subsaturating concentrations of NADPH, on the enzyme activity was determined. As the [NADP]:[NADPH] ratio increased, progressively increasing inhibition of fatty acid synthase activity was observed; 50% inhibition was obtained at a ratio of 1 (Fig. 1).

Since two NADP-dependent reductase activities are involved in fatty acid synthesis, the effect of the [NADP]:[NADPH] ratio on these component activities was also measured. These reductase activities were less severely inhibited than the overall activity of the enzyme by increasing the [NADP]:[NADPH] ratio. The inhibition by NADP of all these three activities was competitive with respect to NADPH (Fig. 2) with $K_I$ values of 6 $\mu$M, 40 $\mu$M, and 260 $\mu$M for overall fatty acid synthase, ketoreductase, and enoyl reductase activities, respectively. In fact, the ratio of the present $K_I$ values of NADP for the enoyl reductase and ketoreductase is nearly the same as the inverse ratio of the $K_I$ values of the two sites for NADPH binding. The higher $K_I$ obtained for the enoyl reductase is consistent with the previously reported affinity of the enoyl reductase for NADPH (10).

The very low $K_I$ value obtained for the overall activity compared to those obtained for the two reductase activities together with the fact that the reductases are not the rate-limiting steps in the overall synthesis of fatty acids suggested that the inhibition by NADP of the overall activity was not entirely due to the inhibition of the two reductases. Therefore, all other component activities of fatty acid synthase were examined for possible inhibition by NADP. Acetyl transacylase, malonyl transacylase, enoyl hydratase, and thioesterase activities were not significantly affected by up to 100 $\mu$M NADP. On the other hand, NADP severely inhibited the condensation reaction; for example, 2 $\mu$M NADP inhibited the condensation activity by more than 50% (Fig. 3A). The extent of inhibition of the condensation reaction by increasing concentrations of NADP was determined by both spectrophotometric (14) and the $^{14}$CO$_2$ exchange assays (16), and the results were identical (Fig. 3A). In the spectrophotometric assay where acetyl-CoA and malonyl-CoA were used as substrates, typical Michaelis-Menten kinetics was observed, and double reciprocal plots showed that NADP inhibition ($K_i = 1.4$ $\mu$M) was noncompetitive with respect to malonyl-CoA concentration (Fig. 3B). Due to the high background rate observed without added acetyl-CoA, the inhibition pattern with varying concentrations of acetyl-CoA could not be determined. In the $^{14}$CO$_2$ exchange assay a sigmoidal saturation pattern (Fig. 4A) was observed for malonyl-CoA as previously observed for fatty acid synthase from pigeon liver (15). However, NADP inhibited the reaction in a concentration-dependent manner at saturating concentrations of malonyl-CoA. Hexanoyl-CoA showed a typical substrate saturation pattern, and NADP inhibited the reaction even at thrice the saturation concentration of hexanoyl-CoA (Fig. 4B). Linear double reciprocal plots showed that NADP inhibition ($K_i = 2.5$ $\mu$M) was noncompetitive with respect to hexanoyl-CoA. Triacetic acid lactone synthesis by pigeon liver fatty acid synthase was found to be

![Fig. 1. Effect of the [NADP]:[NADPH] ratio on enoyl reductase (ER), ketoreductase (KR), and overall activity (FAS) of fatty acid synthase. Concentrations of NADPH (15 $\mu$M) and fatty acid synthase (6.2 $\mu$M) were kept constant and that of NADP varied from 0-75 $\mu$M. All assays were done in 100 mM sodium phosphate buffer, pH 7.0, containing 1 mM EDTA and 1 mM dithioerythritol and at saturating concentrations of all other substrates. Specific activities of fatty acid synthase, ketoreductase, and enoyl reductase were 0.6, 1.4, and 0.04 $\mu$mol/min/mg of protein, respectively.](image1)

![Fig. 2. Double reciprocal plots with NADPH as the variable substrate for overall fatty acid synthase, ketoreductase, and enoyl reductase activities in the presence of NADP. Concentrations of enzyme in the assays were 6.2 nm, 5.5 nm, and 55 nm for overall fatty acid synthase, ketoreductase, and enoyl reductase activities, respectively; NADP concentrations are shown on each line.](image2)
NADP binds at one of the NADPH binding sites (one of the
respect to NADPH also strongly suggests that the inhibitory
overall activity of fatty acid synthase is competitive with
the observation that the inhibitory effect of NADP on the
condensation reaction is dimerization (1). In the case of fatty acid synthase, activity was measured by both spectrophotometric assay (A) and $^{13}$C₂O₂ exchange assay (B). Double reciprocal plots for condensation activity with varying concentrations of malonyl-CoA at fixed concentrations of acetyl-CoA (60 μM) and enzyme (0.9 μM) using the spectrophotometric assay. NADP concentrations are shown on each line.

FIG. 3. Inhibition of condensation activity of fatty acid synthase by NADP. A, concentration dependence of inhibition by NADP of native fatty acid synthase (FAS), fatty acid synthase modified with pyridoxal phosphate in the presence of NADPH (NADPH/PLP), and fatty acid synthase modified with pyridoxal phosphate (PLP-FAS). In the case of fatty acid synthase, activity was measured by both spectrophotometric assay (A) and $^{13}$C₂O₂ exchange assay (B). Double reciprocal plots for condensation activity with varying concentrations of malonyl-CoA at fixed concentrations of acetyl-CoA (60 μM) and enzyme (0.9 μM) using the spectrophotometric assay. NADP concentrations are shown on each line.

FIG. 4. Effect of NADP on condensation ($^{13}$C₂O₂-exchange) activity of fatty acid synthase with varying concentrations of malonyl-CoA and hexanoyl-CoA. NADP concentrations are shown on each line. A, assays were done at concentrations of 2.0 mM CoA, 0.6 mM hexanoyl-CoA, 35 mM NaH$^{13}$C₂O₂, and 0.5 μM enzyme at varying concentrations of malonyl-CoA. B, same as A, but with a fixed concentration of malonyl-CoA (0.2 mM) and varying concentrations of hexanoyl-CoA. Inset, the double reciprocal plot after subtracting the background rate obtained without any added hexanoyl-CoA.

The severe inhibition of the condensation reaction by low concentrations of NADP suggests that this inhibitory binding site has a fairly high affinity for NADP. Since enoyl reductase is known to have a high affinity (K_s = 1.3 μM) for NADPH, it appeared possible that this inhibitory binding of NADP is at this site. Since pyridoxal phosphate is known to actively inhibit NADPH binding at the enoyl reductase site of fatty acid synthase (5, 10), the effect of NADP on the condensing activity of the pyridoxal phosphate-modified enzyme was determined. The condensing activity of the modified enzyme was not affected by NADP even at concentrations which severely inhibited (~80%) the condensing activity of native fatty acid synthase (Fig. 3A). Furthermore, pyridoxal phosphate treatment of the enzyme in the presence of NADP did not abolish the NADP inhibition of the condensing activity. Since pyridoxal phosphate modification does not affect the condensing activity and other component activities of fatty acid synthase other than enoyl reductase, this result strongly suggests that NADP binding at the enoyl reductase domain is responsible for the inhibition of the condensing activity. Since pyridoxal phosphate modification of only a single lysine residue per peptide is prevented by NADPH (5), the above results suggest that the inhibitory binding site of NADP is the same as the NADPH binding site at the enoyl reductase domain. Obviously, NADP binding at the enoyl reductase site brings about conformational changes which adversely affect the active site of the condensation domain.

To test the structural features of NADP relevant to the inhibition of the condensing activity various analogs were tested for their effect on this activity. NAD, 2'-AMP, 2'-monophospho-adenosine 5'-diphosphoribose, and nicotinamide ribose monophosphate did not inhibit the condensing activity even at concentrations exceeding 50 times the K_s value for NADP (Table I). Since the 2'-phosphate of NADP is known to be involved in the binding to the enoyl reductase site (4, 5), it is not surprising that NAD and nicotinamide mononucleotide failed to inhibit the condensing activity. In view of the finding that 2'-AMP and 2'-monophosphoadenosine 5'-diphosphoribose bind to the NADP binding site of enoyl reductase domain (5), their inability to inhibit condensing activity suggests that the nicotinamide moiety is essential for the inhibitory effect of NADP.

TABLE I

<table>
<thead>
<tr>
<th>Addition</th>
<th>Concentration (μM)</th>
<th>% Activity</th>
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<tr>
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<td>100</td>
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<tr>
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<td></td>
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<td>100</td>
<td>98</td>
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<tr>
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</tr>
<tr>
<td>2'-Monophosphoadenosine</td>
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</tr>
<tr>
<td>5'-diphosphoribose</td>
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<td>104</td>
</tr>
<tr>
<td>β-Nicotinamide ribose monophosphate</td>
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* Spectrophotometric assay was used to monitor the activity.
studies were conducted on pyridoxal phosphate-modified enzyme. Ultracentrifugation of the native enzyme dialyzed against low ionic strength phosphate buffer showed that ~70% of the enzyme was dissociated into monomers (Fig. 5A). Upon addition of NADPH virtually all of the enzyme dimerized (Fig. 5B). Pyridoxal phosphate-modified enzyme also dissociated in low ionic strength buffer (Fig. 5C), but addition of NADPH to this dissociated enzyme did not cause dimerization (Fig. 5D). That the ability of the enzyme to dimerize was not lost by pyridoxal phosphate modification was shown by the observation that the addition of 0.2 M KCl to nearly completely dissociated enzyme caused virtually complete dimerization (Fig. 5F) although NADPH could not cause dimerization (Fig. 5E). As previously noted with fatty acid synthases from other vertebrates, dissociation of the present enzyme caused inactivation of condensing activity with both native and pyridoxal phosphate-modified enzyme. The condensing activity of both enzyme preparations was fully recovered upon dimerization with KCl. These results suggest that the dimerization triggered by NADPH is caused by the binding of this nucleotide at the enoyl reductase domain of fatty acid synthase.

**Effect of NADPH Binding at the Enoyl Reductase Domain on the Proteolysis of Fatty Acid Synthase**—Proteolysis of several enzymes can be slowed down by substrates, and this was retained. Other substrates such as malonyl-CoA and acetyl-CoA did not show any significant effect on the rate of proteolysis. That the ability of the enzyme to dimerize was not lost by pyridoxal phosphate modification was shown by the observation that the addition of 0.2 M KCl to nearly completely dissociated enzyme caused virtually complete dimerization (Fig. 5F) although NADPH could not cause dimerization (Fig. 5E). As previously noted with fatty acid synthases from other vertebrates, dissociation of the present enzyme caused inactivation of condensing activity with both native and pyridoxal phosphate-modified enzyme. The condensing activity of both enzyme preparations was fully recovered upon dimerization with KCl. These results suggest that the dimerization triggered by NADPH is caused by the binding of this nucleotide at the enoyl reductase domain of fatty acid synthase.

Since pyridoxal phosphate-modified enzyme does not have any overall activity of fatty acid synthase, the protection of overall activity by NADPH against proteolysis could not be examined and, therefore, ketoreductase activity of the enzyme was used as a measure of proteolytic destruction of the enzyme. In the presence of NADPH, ketoreductase activity was totally resistant to trypsin treatment whereas in the absence of NADPH this activity was destroyed (Fig. 6B). The ketoreductase activity of pyridoxal phosphate-modified enzyme was just as susceptible to trypsin treatment as that of the native enzyme (Fig. 6C). Protection of the ketoreductase activity against trypsin by NADPH was very much less in the pyridoxal phosphate-treated enzyme than in the native enzyme. Similar results were obtained by monitoring ketoreductase activity of native and pyridoxal phosphate-modified enzyme during clostripain treatment in the presence and absence of NADPH. Furthermore, fatty acid synthase modified with pyridoxal phosphate in the presence of NADPH was resistant to proteolysis in the presence of NADPH (Fig. 6D). Malonyl-CoA and acetyl-CoA did not protect the enzyme from trypsin cleavage (Fig. 6, B, C, and D). These results further illustrate the role of the NADPH binding site in the enoyl reductase domain in maintaining the functional conformational integrity of fatty acid synthase.

**DISCUSSION**

The results presented in this paper show that NADP inhibits the condensing activity by binding to the enoyl reductase domain of fatty acid synthase. Since this binding does not significantly affect the other component activities of the enzyme, it appears probable that the major conformational change brought about by NADP is in the condensation domain. Such a conformational change might inhibit the condensation reaction by altering the juxtapositioning of the —SH group of the phosphopantetheine and that of the condensing enzyme, which was recently suggested to be required for the condensing activity (19). On the basis of intermonomer cross-linking between the two —SH groups by dibromoacetone it was suggested that the —SH group of phosphopantetheine of one peptide and the thiol of the condensing enzyme of the other peptide are in close proximity and function in conjunction with each other in the condensation process (19). The inhibition of condensation by NADP could be the result of a conformational change which caused an alteration in the juxtapositioning of the —SH groups. However, even in the presence of up to 100 mM NADP dibromoacetone cross-linked the two peptides of the present enzyme while derivatization of a single —SH/monomer (presumably the —SH group of the condensing enzyme) with iodoacetamide prevented this cross-linking. Therefore, if dibromoacetone, in fact, cross-links the two specific —SH groups, NADP does not perturb their juxtapositioning. In any case, the regulation of the condensing activity by NADP binding in the enoyl reductase domain provides the first example of functional regulation by an interdomain interaction in fatty acid synthase.

The proximity of the enoyl reductase domain and the condensation domain and the functional interaction between the two domains suggested by the present results could provide an explanation for the following observations. (a) The crotonyl-CoA reduction catalyzed by the fatty acid synthase from bovine mammary glands was suggested to occur while the crotonyl group is bound to the primer binding site (20) indicating the proximity of this site with the active site of enoyl reductase. (b) In yeast fatty acid synthase in which the condensation domain and enoyl reductase domain are located in two different peptides, condensing activity was found to be higher in mutants with defective enoyl reductase domains (21) indicating possible functional interaction between the two

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domains in a manner similar to that suggested by the present results. (c) In the acyl carrier protein-dependent multicomponent synthase of *Euglena gracilis*, enoyl reductase tends to associate with the condensing enzyme even in cell-free preparations (22). This association could provide a means for regulating the condensing activity by NADP binding to the enoyl reductase. If such an association is a general feature of multicomponent fatty acid synthases, such as that found in *Escherichia coli*, the NADP regulation discovered in the present vetebrate enzyme could be a regulatory feature acquired early in evolution. This possibility is being explored at the present time.

Of the two NADPH binding sites present in each peptide, the enoyl reductase site has a substantially higher affinity for this nucleotide than does the ketoreductase site (10). Therefore, it is reasonable that the high affinity site would be involved in the physiological regulation of the enzyme activity by this nucleotide. The present results suggest that NADPH binding at the enoyl reductase domain could regulate fatty acid synthase activity in three different ways under physiological conditions. (a) NADP binds to the enoyl reductase domain and thus inhibits the first and rate-limiting reaction involved in the process of fatty acid synthesis. Although the competition between NADPH and NADP for this binding site was revealed by the kinetic studies, it is not possible to directly measure the effect of the NADP:NADPH ratio on the condensation activity. However, it would seem reasonable to suggest that the oxidation-reduction state of the cell regulates fatty acid synthase at the condensation step by the pyridine nucleotide binding at the enoyl reductase domain. (b) Catalytically inactive monomeric fatty acid synthase would be converted into the catalytically active dimeric form by NADPH binding at the enoyl reductase domain. (c) NADPH binding to the enoyl reductase domain could regulate the turnover of the enzyme by inhibiting proteolysis as suggested by the results obtained with clostripain and trypsin. All these three modes of regulation together illustrate how the enoyl reductase domain plays a central role in regulating fatty acid synthase in accordance with the oxidation-reduction state (NADP:NADPH ratio) of the cell.

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**REFERENCES**