Modification of mammalian fatty acid synthetase activity by NADP

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Experiments are described which show that the mammalian fatty acid synthetase, in the presence of NADP, synthesizes stoichiometric amounts of enzyme-bound acetoacetyl moieties. The acetoacetyl moieties can neither undergo the normal transfer reaction to a CoA acceptor, nor participate in the normal reaction sequence once NADPH is made available. Our results indicate that, since it is the product of the condensation reaction which accumulates on the inhibited enzyme, the previously held view that NADP inhibits the condensation step in fatty acid synthesis is probably incorrect.

Fatty acid synthetase *NADP* *Acetoacetyl-enzyme*

1. INTRODUCTION

The vertebrate fatty acid synthetases are composed of two identical polyfunctional polypeptides each of about 250 kDa [1]. Two pairs of NADPH binding sites, corresponding to the ketoreductase and enoyl reductase domains are present on the dimer [2–4]. The binding of NADPH to the reductase domains is inhibited competitively by NADP and it has been suggested that the activity of fatty acid synthetase in vivo might be influenced by the oxidation-reduction potential of the cell [5]. However the effects of pyridine nucleotide binding are not limited to the activity of the two reductases and it has been suggested that pyridine nucleotides may stabilize the fatty acid synthetase by preventing dissociation into monomers and by protecting the enzyme from proteolysis [5,6]. Some of these effects may be mediated by interdomain reactions promoted by nucleotide binding at the enoyl reductase domain [5]. In the course of our studies on fatty acid synthetase we inadvertently discovered a novel inhibitory effect of NADP which is mediated by the presence of a condensation product on the enzyme. Results of this study indicate that the mechanism of interaction of pyridine nucleotides with fatty acid synthetase requires careful reevaluation.

2. MATERIALS AND METHODS

Acetoacetyl-CoA was assayed [7] using β-hydroxyacyl-CoA dehydrogenase (Sigma), malonyl-CoA using fatty acid synthetase [8], and acetoacetate [9] using β-hydroxybutyrate dehydrogenase (Calbiochem). All three spectrophotometric assays were unaffected by components carried over from preincubation systems, as verified using the appropriate internal standards. Fatty acid synthetase was purified from rat liver [10] and assayed spectrophotometrically [8] at 25°C; units of activity are nmol NADPH oxidized per min. Substrate binding to fatty acid synthetase was estimated by acid precipitation on Millipore filters [11]. Acetyl-CoA and malonyl-CoA were purified by high pressure liquid chromatography [12].

3. RESULTS

Preincubation of fatty acid synthetase with NADP, acetyl-CoA and malonyl-CoA diminished the initial rate in subsequent fatty acid synthetase

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assays to less than 10% of the normal value. During the fatty acid synthetase assay, the reaction rate continued to increase for about 2 min reaching a value corresponding to about 50% of the normal initial rate (fig.1). Omission of any one of the components of the preincubation system abolished the inhibitive phenomenon. The concentration of NADP required to produce the inhibition was surprisingly low (NADP:enzyme molar ratio of 2.5:1). The requirement for both acetyl-CoA and malonyl-CoA suggested that perhaps the presence of a condensation product on the enzyme was necessary to produce the NADP inhibitive effect. Indeed acetoacetyl-CoA, which can function as a substrate for mammalian fatty acid synthetases [14], could substitute effectively for acetyl-CoA and malonyl-CoA in the preincubation system with retention of the NADP inhibitive phenomenon (fig.1). Acetoacetate was ineffective (not shown). Confirmation of the identity of the condensation product bound to the inhibited enzyme was initially obtained chromatographically (fig.2). Recovery of the carrier acetoacetate, estimated enzymatically, was only 44% and a significant amount of radioactivity co-eluted with the unbound acetone carrier, indicating that a substantial fraction of the acetoacetate had probably decarboxylated under the conditions of isolation. Independent and quantitative identification of the radiolabeled acetoacetyl-enzyme condensation product was obtained by intentional decarboxylation to acetone and formation of the semicarbazone derivative (table 1). The results indicated that about 1.5 mol acetyl moietites and

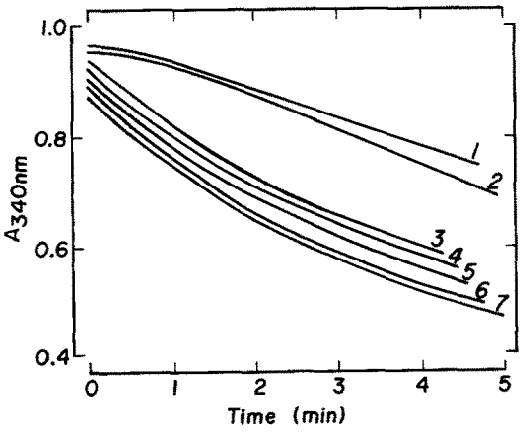

Fig.1. Inhibition of fatty acid synthetase induced by preincubation with NADP and substrates. Fatty acid synthetase (2 μM) was incubated for 1 min at 20°C in 0.1 M potassium phosphate buffer (pH 7.0) containing various combinations of substrates (50 μM acetyl-CoA; 50 μM malonyl-CoA; 10 μM acetoacetyl-CoA; 5 μM NADP) and an aliquot (10 μg enzyme) was assayed for fatty acid synthetase activity at 25°C. Initial rates were computed by a Gilford Response spectrophotometer. The initial absorbances of the various reaction mixtures have been offset for clarity. The substrates included in the preincubations and the subsequent initial rates observed (in parentheses) were: 1, malonyl-CoA, acetyl-CoA and NADP (130 U/mg); 2, acetoacetyl-CoA and NADP (50 U/mg); 3, malonyl-CoA and acetyl-CoA (1000 U/mg); 4, acetyl-CoA and NADP (1040 U/mg); 5, malonyl-CoA and NADP (900 U/mg); 6, NADP only (1050 U/mg); 7, acetoacetyl-CoA only (910 U/mg).

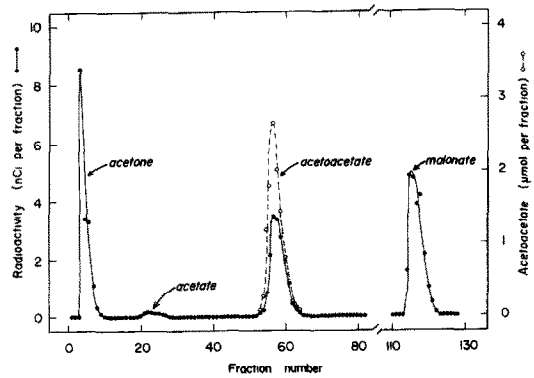

Fig.2. Identification of acetoacetyl moiety bound to fatty acid synthetase inhibited with NADP. Fatty acid synthetase (4 μM) was preincubated in 0.25 ml of 130 mM potassium phosphate buffer (pH 6.6) containing 25 μM [2-^{14}C]malonyl-CoA (58 Ci/mol), 25 μM acetyl-CoA and 25 μM NADP for 2.5 min at 20°C. Protein was precipitated and washed with ethanol (50%, v/v, final concentration) and deacylated in 0.2 M NaOH containing carrier acetone, acetoacetic acid and malonic acid. The solution was neutralized with HCl and applied to a column (8 × 1 cm) of Dowex 1-X8 (200–400 mesh) equilibrated with 0.01 M formic acid. The column was developed with 5 ml of 0.01 M formic acid followed by a 80-ml gradient of formic acid (0.01–0.5 M) and 40 ml of 0.2 M HCl. Fractions were collected and immediately neutralized to pH 8.5 with $KHCO_3$. Radioactivity (●—●) was estimated by liquid scintillation spectrometry and acetoacetate (○---○) by enzymatic assay. Location of the acetone, acetate, acetoacetate and malonate zones was confirmed by chromatography of authentic standards.

Table 1

Stoichiometry of formation of acetoacetyl moieties by fatty acid synthetase in the presence of NADP

Radiolabeled substrate	Incubation time (min)	Enzyme-bound substrate recovered as acetone semicarbazone (mol/mol enzyme)	Unbound substrate recovered as acetone semicarbazone (mol/mol enzyme)
Acetyl-CoA	2	1.39	1.15
Malonyl-CoA	2	1.53	1.11
Acetyl-CoA	10	1.50	1.26
Malonyl-CoA	10	1.48	1.40

Fatty acid synthetase (4 μM) was incubated at 20°C in 0.25 ml of 130 mM potassium phosphate buffer (pH 6.6) containing 25 μM acetyl-CoA, malonyl-CoA and NADP. Radiolabel was present on only one substrate (58 Ci/mol). Protein was separated from the reaction medium by precipitation with ethanol (50%, v/v, final concentration) and dissolved in 0.2 M NaOH. Carrier acetone was added and acyl-thioesters in the precipitate and supernatant were hydrolyzed at 90°C for 60 min. Subsequently decarboxylation of acetoacetate was carried out in 1.6 M sulfuric acid, 20°C for 60 min. The solutions were adjusted to pH ~6 with NaOH. Sodium acetate (0.5 M final concentration) and a 50% molar excess of semicarbazide (1.5 M final concentration) were added. Acetone semicarbazones were recrystallized to constant specific radioactivity from water

1.5 mol malonyl moieties had condensed on the enzyme to form 1.5 mol acetoacetyl-enzyme. A small amount of acetoacetate was released from the enzyme. The stoichiometry of acetoacetyl-fatty acid synthetase formation was also estimated by first incubating fatty acid synthetase (1 μM) with [1-^{14}C]acetyl-CoA (50 μM, 6.74 Ci/mol), malonyl-CoA (50 μM) and NADP (0.2 mM). Unreacted malonyl and acetyl moieties were then removed from the enzyme by addition of CoA (0.5 mM) as an acceptor [11]. The residual, non-exchangeable radioactivity accounted for 1.8 mol acetyl moieties (as acetoacetate) per mol of enzyme. Since the enzyme preparation contained 1.7 mol 4'-phosphopantetheine per mol dimer [13], the NADP-inhibited enzyme apparently contains an acetoacetyl moiety at both active centers.

In the absence of reducing equivalents we anticipated that the acetoacetyl moiety either might be transferred from the enzyme to an acceptor such as CoA [14] or might participate in a second condensation reaction resulting in the formation of triacetic lactone [15]. When fatty acid synthetase was incubated with acetyl-CoA and malonyl-CoA, in the absence of pyridine nucleotides, we found that the rate of acetoacetyl-CoA synthesis, 51.5 ± 2.1 nmol/min per mg fatty acid synthetase was exactly balanced by the rate of disappearance of malonyl-CoA, 52.0 ± 4.2 nmol/min per mg fatty acid synthetase. In the presence of NADP however, no acetoacetyl-CoA synthesis was detected with the enzymatic assay and the utilization of malonyl-CoA was sufficient to account for the synthesis of only stoichiometric amounts of acetoacetyl-enzyme. This result indicated that NADP blocks the transfer of acetoacetyl moieties from enzyme to CoA.

4. DISCUSSION

Two lines of evidence indicate that, in contrast to the avian enzyme, the mammalian fatty acid synthetase does not catalyze the synthesis of triacetic lactone in the absence of NADPH. First, the mammalian enzyme can dispose of the enzyme-bound acetoacetyl moiety by transfer back to a CoA acceptor, as shown here; the transferase component of the avian enzyme is unable to utilize

acetoacetyl moieties in this way [14]. Thus in the absence of NADPH, acetoacetyl moieties probably do not remain on the enzyme for participation in a second condensation reaction. Secondly, we observed a perfect balance in malonyl-CoA utilized and acetoacetyl-CoA synthesized, which clearly does not allow for the formation of any other product such as triacetic lactone. In the presence of NADP, the utilization of malonyl-CoA was insufficient to account for any synthesis and release of triacetic acid lactone. Furthermore the enzyme-bound intermediate present on the NADP-inhibited enzyme was identified as an acetoacetyl moiety.

Our results show that the inhibitive effect of NADP on fatty acid synthetase is linked to the presence of a condensation product on the enzyme. Thus, when fatty acid synthetase is preincubated separately with NADP and only one of the two substrates, subsequent exposure to NADPH in the fatty acid synthetase assay results in the immediate replacement of the NADP with NADPH and the immediate restoration of full activity. Only when an acetoacetyl moiety is simultaneously present on the enzyme will NADP inhibit fatty acid synthetase activity. Previous studies on the effects of NADP on fatty acid synthetase activity have not considered the effect of the presence of substrates. Thus, Poulose and Kolattukudy [5] concluded that NADP-inhibition of the overall activity of the avian fatty acid synthetase could result from competitive inhibition of the NADPH-dependent keto- and enoyl-reductase reactions. In addition they suggested that NADP binding at the enoyl reductase domain resulted in inhibition of the condensation reaction via an interdomain interaction. Our results indicate that since, in the presence of NADP, it is the condensation product which accumulates on the enzyme either this interpretation is incorrect or there is a fundamental difference in the effect of NADP on the avian and mammalian enzymes. Certainly it is apparent that in the presence of NADP, the acetoacetyl condensation product cannot participate in the normal translocation reaction catalyzed by the mammalian enzyme which results in the formation of acetoacetyl-CoA. Alternative explanations for the NADP effect are now being evaluated.

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