Effect of arginine modifying reagents on pigeon liver fatty acid synthetase: Evidence for the presence of essential arginine residues at the β-ketoacyl reductase and enoyl-CoA reductase domain

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Received 10 June 1999; revised 14 September 1999

Pigeon liver fatty acid synthetase was inactivated by arginine modifying reagent, phenylglyoxal and 2,3-butanedione. The inactivation of overall fatty acid synthetase was accompanied by the loss of β-ketoacyl reductase and enoyl-CoA reductase activity. The inactivation followed a pseudo-first order kinetics and sum of the second order rate constants for the two reductase reactions equaled that for the synthetase reaction. Inactivation of all three activities was prevented by NADPH or its analogs 2′,5′-ADP and 2′-AMP but not by the corresponding nucleotides containing the 5′-phosphate. These results suggest that binding of NADPH to fatty acid synthetase involves specific interaction of the 2′-phosphate with the guanidino group of arginine residues at the active site of the two reductases. pH-dependent inactivation by phenylglyoxal indicated that a group with a pKa 7.5 is involved in the loss of enzyme activity. Stoichiometric results showed that 4 out of 164 arginine residues per enzyme molecule were essential for the enzyme activity.

Pigeon liver fatty acid synthetase is a multifunctional enzyme complex which catalyses the synthesis of palmitic acid by seven different sequential enzymatic reactions. Among these reactions fatty acid synthetase catalyses two NADPH dependent reduction steps, β-Ketoacyl reductase catalyses the first reduction and enoyl-CoA reductase catalyses the second reduction step. Since arginine is known to be involved in the binding of anionic substrates to several enzymes, so it is possible that arginine residues may be involved in the binding of NADPH to FAS.

To test this possibility the effect of two arginine specific reagents phenylglyoxal and 2,3-butanedione was examined. In the present study we have shown the presence of arginine residues at the active site of β-ketoacyl reductase and enoyl-CoA reductase domain of pigeon liver FAS which interact specifically with the 2′-phosphate of NADPH.

Materials and Methods

Materials

Acetyl-CoA, malonyl-CoA, NADPH, phenylglyoxal, 2,3-butanedione, 5,5-dithiobis (2-nitrobenzoic acid), 2′-AMP, 2′-ADP, 5′-AMP and buffer components (Sigma), Sephadex G-25 (Pharmacia), dithiothreitol (DTT) (Calbiochem) were procured from the above designated sources. All other chemicals were of highest purity grade commercially available. Distilled water from Millipore Milli Q system was used in all experiments. The substrates, S-crotonyl-N-acetyl cysteamine and S-acetoacetyl-N-acetyl cysteamine were synthesized by the method as described to measure reductase activity.

Methods

Purification of pigeon liver FAS and assay of overall FAS and reductase activities

Pigeon liver fatty acid synthetase was purified and assayed spectrophotometrically by the decrease in the absorbance of NADPH at 340 nm on UV-vis spectrophotometer (Shimadzu Model UV-160A) by the method of Muesing and Porter. The enzyme was subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to the standard method of Laemmli and exhibited a single band. Assays for β-ketoacyl and enoyl-CoA reductase activities were carried out spectrophotometrically as described earlier. All measurements were carried out at 25°C.

Modification of FAS with phenylglyoxal and 2,3 butanedione

The stock solution of phenylglyoxal was prepared by first dissolving the crystals in 1% ethanol and then
diluting the solution with water. Solutions of NADPH and phenylglyoxal were prepared fresh daily and concentrations were determined spectrophotometrically using extinction coefficients of 6.22 \times 10^{-3} \text{ cm}^{-1} \text{ M}^{-1} at 340 nm, and 12.4 \times 10^{-3} \text{ cm}^{-1} \text{ M}^{-1} at 253 nm respectively. Inactivation of FAS was carried out at 25°C in 0.2 M potassium phosphate buffer (pH 7.0), 1 mM EDTA, 1 mM DTT and 80 mM NH_{4}HCO_{3} at a protein concentration of 3-12 \mu M, specific activity 35 nmoles palmitate per min per mg of protein with various concentrations (1-7 mM) of phenylglyoxal. The incubations were performed in the dark to minimize light-induced decomposition of reagent. Modification with various concentrations of 2,3-butanedione (10-40 mM) was carried out in 20 mM borate buffer containing 200 mM KCl, 1 mM DTT and 1 mM EDTA at pH 7.5 and 25°C. At different time intervals, aliquots were withdrawn and assayed for overall FAS, enoyl-CoA and \beta-ketoacyl reductase activity. Controls, in duplicate, without phenylglyoxal and 2,3-butanedione were run concurrently.

**Effect of substrates and substrate analogs on inactivation of FAS by phenylglyoxal**

Protection against inactivation by phenylglyoxal was done by adding the protecting agents to the enzyme solution before the addition of the modifier. The enzyme (12 \mu M) was incubated with the protecting ligands for 10 min prior to the addition of phenylglyoxal (3 mM). After 30 min the overall FAS and enoyl-CoA and \beta-ketoacyl reductase activity was determined.

**The pH rate profile of the inhibition of FAS by phenylglyoxal**

The decrease in the enzyme activity was monitored at different pH values. The pseudo-first-order rate constants were obtained by plotting the data on a semi-logarithmic scale. The second-order rate constant was calculated by dividing the pseudo-first-order rate constant by the concentration of phenylglyoxal. The pH effect on the enzyme inactivation was analysed according to the following equation:

\[
\frac{1}{k} = \left[ \frac{1}{k_{\max}} - \frac{K}{[H^{+}]} + \frac{1}{k_{\max}} \right]
\]

Where \( k \) is the second-order rate constant for each pH, \( k_{\max} \) is the maximal second-order rate constant, \( K \) is the ionization constant of the group involved in the catalysis and \([H^{+}]\) is the hydrogen ion concentration.

**Stoichiometry of reaction of phenylglyoxal with FAS**

The number of arginine residues modified was determined by incubating the enzyme (4 mg/ml) with 2.5 mM phenylglyoxal for different time intervals. After incubation for the indicated time intervals the synthetase activity was determined and the reaction was terminated with 10 mM arginine. Excess reagent was removed by column centrifugation method according to Penefsky and the absorbance was measured at 250 nm using the extinction coefficient of 11 \times 10^{3} \text{ cm}^{-1} \text{ M}^{-1} (ref. 15, 16).

**Results and Discussion**

Incubation of the pigeon liver FAS with phenylglyoxal resulted in the time dependent loss of synthetase, \beta-ketoacyl reductase and enoyl-CoA reductase activities and inactivation was found to be irreversible. The inactivation followed pseudo-first-order kinetics, as indicated by typical semi-log plots of activity vs time (Fig. 1, 2 and 3) which were linear up to 90% inactivation. Second order rate constants \((k_{2})\) were obtained from slopes of linear plots of first-order rate constant \((k_{1})\) vs reagent concentration (Fig. 1a) and inset Fig. 2a and 3a were 2.4 \times 10^{-2}, 1.1 \times 10^{-2} and 1.3 \times 10^{-2} \text{ mM}^{-1} \text{ min}^{-1} for overall FAS, enoyl and \beta-ketoacyl reductase respectively. The reaction order \((n)\) with respect to reagent was determined from the slope of a double log-plot according to the equation:

\[
\log k_{i} = \log k_{2} + n \log [R]
\]

Where \([R]\) represents reagent concentration. Double-log plots of \(k_{2}\) as a function of reagent concentration (Fig. 1b) and inset Fig. 2b and 3b were also linear, yielding slopes of 1.10, 1.15 and 1.04, respectively which is close to one indicating, a reaction order of one with respect to reagent for each of the activities. The second order rate constants for inactivation of \beta-ketoacyl reductase and enoyl-CoA reductase were nearly identical, and the sum of these values equaled to the rate constant for the inactivation of overall synthetase activity. This behaviour is in accordance with a mechanism whereby phenylglyoxal reacts simultaneously with residue(s) at or near the active sites of both reductases and that modification of either reductase domain results in the loss of synthetase activity. This mechanism is also supported by a kinetic order of one with respect to reagent for loss of synthetase activity.
2,3-Butanedione, another widely used reagent, which in borate buffer modifies arginine residues in proteins\textsuperscript{1,7} was also used for modification of FAS activity and this inactivation was dependent on time and concentration of the reagent. The inactivation followed pseudo-first order kinetics as indicated by semi-log plots of activity versus time which was linear (Fig. 4). The second order rate constant \( (k_2) \) was obtained from slopes of linear plots of first-order rate constant \( (k_1) \) vs reagent concentration (Fig. 4, inset) and was \( 1.2 \times 10^{-3} \) mM\textsuperscript{-1} min\textsuperscript{-1}. 2,3-Butanedione also inactivated ketoacyl and enoyl reductase activities of FAS but the overall FAS activity was more sensitive to inactivation than the two reductase activities (data not shown).

In order to know the site of inhibition protection studies were performed using substrates and substrate analogs. As shown in Table 1 acetyl-CoA and malo-
N-acyl-CoA had no effect whereas NADPH or NADP provided significant protection against inactivation of synthetase, β-ketoacyl reductase and enoyl-CoA reductase activities. Similar effects were observed for 2-phosphate derivatives of adenine nucleotides such as 2'-AMP or 2',5'-ADP but not for the 5'-phosphate derivatives 5'-AMP or 5'-ADP. NAD⁺ had no effect on the loss of synthetase activity. These results suggest that binding of NADPH to FAS involves specific interaction of the 2'-phosphate with the guanidino group of arginine residue at the active site of the two reductases. Overall FAS, ketoacyl reductase and enoyl reductase activities showed similar protection by 2,3-butanedione by only NADPH or NADP and its 2'-phosphate-containing analogs (data not shown).
Although phenylglyoxal is highly specific for arginine residues\(^\text{11}\) other amino acids such as cysteine and to a lesser extent histidine and lysine were known to undergo reaction under certain harsh conditions\(^\text{12}\). In a separate experiment, the essential SH groups of FAS were reversibly blocked by incubating 3 \(\mu\)M enzyme with 30 \(\mu\)M 5,5'-dithiobis-(2-nitrobenzoic acid) for 35 sec at room temperature. Following removal of excess disulfide reagent by gel filtration, the enzyme derivative was reacted with 2 \(mM\) phenylglyoxal and timed samples were assayed for synthetase activity 30 min after the addition of 50 \(mM\) DTT. The first order rate constant of inactivation of 0.02 min\(^{-1}\) was comparable to that obtained for the unblocked enzyme (\(k_1 = 0.0198\) min\(^{-1}\)), indicating that phenylglyoxal inactivation did not involve reaction of essential SH groups.

The inactivation rate of FAS by phenylglyoxal was examined over the pH range 6.0-8.0. The pseudo first order rate constants obtained for the enzyme inactivation were plotted as a function of pH (Fig. 5). A straight line was obtained when 1/k min was plotted against [\(H^+\)] from which a \(pK_a\) value of 7.5 was obtained. This \(pK_a\) value is lower than the usual value (9.5) for the free arginine. The difference in \(pK_a\) may be due to the presence of a hydrophobic environment required for the catalysis.

In order to determine the number of reactive residues involved in inactivation the absorbance of the
diphenylglyoxal adduct with guanidino group of arginine of enzyme molecule was measured at 250 nm at different stages of modification. Number of arginine residues modified were calculated by taking the ratio of concentration of diphenylglyoxal adduct and the enzyme. The concentration of diphenylglyoxal adduct and the enzyme were determined by using the absorbance coefficient of 11000 cm\(^{-1}\) M\(^{-1}\) at 250 nm\(^{-1}\) and Lowry et al.\(^{10}\) respectively. Molecular weight of FAS was taken as 450,000. As shown in Fig. 6, a plot of residual synthetase activity vs arginine residues modified was linear and extrapolated to a value of 4.0, indicating that four arginine residues per enzyme molecule reacted for the complete loss of activity. Therefore it appears that the four arginine residues modified are at the two reductase sites.

It is reasonable to conclude that there is one essential arginine at each of the ketoacyl reductase and enoyl-CoA reductase sites per subunit. The presence of fast-reacting, non-essential residues as seen on the synthetase from goose uropygial gland\(^{10}\) was not observed indicating greater reagent selectivity. This result is similar to the stoichiometry obtained for chicken liver FAS. These results also suggested that each subunit of FAS contains one enoyl-CoA reductase and one \(\beta\)-ketoacyl reductase domain having one arginine residue located at the NADPH binding site. Previous findings have shown that each peptide contains a covalently attached phosphopantetheine and a thioesterase domain\(^{21, 22}\). All these results support the homodimer model of FAS. Further studies are in progress to locate the position of this critical arginine residue at the active site of FAS by isolating the reactive peptides and sequencing after modification with phenylglyoxal.

Acknowledgement
We are thankful to Dr. Leela lyengar for helpful discussion.

References
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