Vol. 164, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 16, 1989 Pages 434-438

NEGATIVE FUNCTIONAL INTERFERENCE BETWEEN TWO ACTIVE CENTERS IS INDICATED IN ANIMAL FATTY ACID SYNTHETASE

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Received August 15, 1989

SUMMARY : When fatty acid synthetase of the Harderian gland of guinea-pig was treated with various amounts of phenylmethanesulfonyl fluoride, the overall activity of the enzyme showed a quadratic decrease with respect to the inhibition degree of the thioesterase activity which was the primary target of inhibition. Moreover, the overall activity per active center of a heterodimer, which was formed between the native monomer and the thioesterase-less monomer, was higher than that of the native enzyme. These results are consistent with the view that the two active centers of the native enzyme exhibit a negative interference to each other.

Animal fatty acid synthetase consists of two giant subunits with multiple active sites (1-6). The subunits are identical to each other and each has seven enzymatic activities and an acyl carrier segment, all of which are involved in the de novo synthesis of fatty acids.

In 1981, Stoops and Wakil (7) proposed a functional model of the chicken liver enzyme. Two subunits were placed in an antiparallel, head-to-tail arrangement in their model , creating two inter-subunit active centers for the synthesis of saturated fatty acids. The model predicted that only the dimeric form of the enzyme would have an overall fatty acid synthesizing activity and its dissociation to monomers should destroy both active centers. According to the model, the two active centers were structurally equivalent but their possible functional relationship has remained unanswered. To answer the question whether the two active centers in the enzyme function independently or alternately, we tried to produce fatty acid synthetase with only one active center in a dimer and compared

0006-291X/89 $1.50

its specific activity with half of the specific activity of the native enzyme. We concluded that the two active centers exhibited a negative functional interference similar to a flip-flop mechanism which was discussed by Lazdunski (8) for the alkaline phosphatase of Esherichia coli though this conclusion was disputed by others (9-11).

MATERIALS AND METHODS

Fatty acid synthetase was purified from the Harderian gland of guinea-pig as described previously (12). The overall activity of the enzyme was measured spectrophotometrically according to Smith and Abraham (3), with modifications as reported by Kitamoto et al. (12). The activity of the thioesterase domain was assayed by a modified procedure of Kolattukudy et al. (13). The reaction mixture consisted of 0.2 M potassium phosphate buffer (pH 7.0), 0.5 mM dithiothreitol, 0.22 mg/ml bovine serum albumin and 50 µM palmitoyl-CoA. One unit of the thioesterase activity was defined as the amount of enzyme required to hydrolyze 1 nmole palmitoyl-CoA in 1 min.

Partial inhibition of fatty acid synthetase with phenylmethanesulfonyl fluoride was done in 0.2 M potassium phosphate buffer (pH 7.0) containing 10 mM dithiothreitol and 1 mM EDTA. The enzyme (0.96 mg/ml) was incubated at 25°C with the inhibitor (0-2.5 mM) in N,N-dimethylformamide. After 1 h, the unreacted inhibitor was removed by passage through a column (0.5 cm x 5.0 cm) of Sephadex G-50 Fine.

Fatty acid synthetase was separated into the thioesterase domains and the thioesterase-less, "core" fragment by incubation with 1/15,000 (w/w) of Pleurotus ostreatus protease B (a gift of Prof. Yoichi Hashimoto, Saitama University) in 0.2 M potassium phosphate buffer (pH 7.0) at 20°C. After 3 hours of incubation, the protease was inactivated by the addition of EDTA at a final concentration of 5 mM. The reaction mixture was subjected to gel filtration on a column (1.9 cm x 64 cm) of Sephacryl S-300 Superfine.

The mixture of native fatty acid synthetase and the core in 0.2 M potassium phosphate buffer (pH 7.0) containing 10 mM dithiothreitol and 1 mM EDTA was incubated at 0°C for 3 days. Under such conditions, 80-90% of the native enzyme was dissociated into subunits and inactivated, whereas 50% of the core was dissociated into monomers. The mixture of dissociated native enzyme and the core was then incubated at 30°C for 3 hours. Under the given conditions, three kinds of dimers, namely, native dimers, core dimers and heterodimers between undigested monomers and core monomers were expected to be formed. When native enzymes and core dimers were dissociated separately and then mixed prior to the reassociation procedure, the results were not affected.

RESULTS AND DISCUSSION

The correlation between overall activity for fatty acid synthesis and thioesterase activity of the partially inhibited enzyme with varying amounts of phenylmethanesulfonyl fluoride is shown in Figure 1. If the two active centers are fully independent, the residual overall activity, y, should be equal to (1-x) as given in a solid in Figure 1, where x is the degree of inhibition of thioesterase activity. Experimental points in Figure 1 were found to fit better to the dotted line

representing the relationship expected for two active centers functioning alternately; i.e., $y = 1-x^2$ (14). The above relationship was derived assuming that an impaired active center was twice as active as each of paired ones in terms of the average turnover number. There is thus an interesting possibility that the two active centers exert a functionally negative interference to each other either acting in a flip-flop manner suggested for alkaline phosphatase (8) or each simply slowing down the rate of the other active center. Similar information as to the relationship between the two active centers can be independently obtained by comparing the overall activity per active center of heterodimers formed as described in the previous section with that of homodimers. The heterodimers must contain one complete active center and one thioesterase-less center, whereas native enzymes contain two active centers. thus when core dimers and native enzyme were mixed and allowed to form heterodimers, the total number of complete active centers in the solution remains constant. Therefore, if the two active centers of the enzyme function independently, an addition of core dimers and subsequent formation of heterodimers should not have any effect on the total activity of the enzyme in the solution. Experimentally, a mixture of core dimer and native enzyme was first cooled and later warmed to effect the heterodimer formation as described above.

Figure 2 shows the regain of the overall activity of fatty acid synthetase during the reassociation process of such mixture. The recovered activity of the reassociated mixture was higher by 20-30% at the plateau level than that of similarly treated native enzyme, a clear indication that a solitary active center has a higher activity than a paired ones. If the two active centers in the native enzyme functioned in a fully flip-flop manner, i.e. with a complete negative interference between them, the activity after reassociation with core should reach 150% in the ordinate scale of Figure 2 (14). The difference may be explained as follows. The reassociation reaction was probably not truly random but produced more core dimers which were less active than assumed because association between core monomers seemed to be stronger than that of the native monomers. It took longer to dissociate the core dimers at $0^{\circ}C$ but the dimerization of core monomers proceeded as rapidly as the native monomers. Another possibility is that the

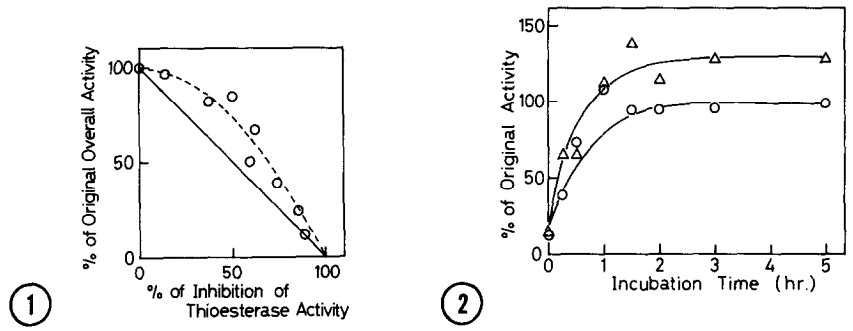

Fig.1 Effect of phenylmethanesulfonyl fluoride to the overall and the thioesterase activities. The thioesterase activity of fatty acid synthetase (0.96 mg/ml) was partially inhibited by 0-2.5 mM phenylmethanesulfonyl fluoride. After unreacted inhibitor was removed, the overall activity of fatty acid synthesis (ordinate) and the thioesterase activity (abscissa) were measured. In this figure, the correlation between the two activities is shown. Solid line: theoretical curve when the two active centers of the enzyme function independently. Dotted line: theoretical curve when they function in a flip-flop manner. Open circles: experimental data.

Fig.2 Activity recovery of the mixture of the native enzyme and the core. Open circles: Equal volumes of the native enzyme (0.34 mg/ml) and 0.2 M potassium phosphate buffer (pH 7.0) containing 10 mM dithiothreitol and 5 mM EDTA were mixed. The mixture was first incubated at 0°C for 10 days, and then transferred to 30°C. Aliquots were removed at indicated intervals and assayed for the overall activity. The pre-dissociation level of the activity was defined as 100%. Open triangles: The core (0.68 mg/ml) in the above buffer was mixed instead of the buffer.

two centers function in a flip-flop manner only partially or, in other words, with a limited negative interference.

If a fixed volume of the core solution of various concentrations was added to the equal volume of native enzyme solution at a constant concentration and the mixture was cooled and warmed as above, the recovered activity was 140% at the highest level, of that of the enzyme solution without added cores (Fig.3). Such results can also be more readily explained

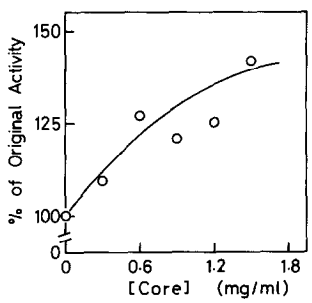

Fig.3 Effect of concentration of the core on the recovery of the overall activity. Equal volumes of the native enzyme (0.57 mg/ml) and the core (0-1.50 mg/ml) were mixed. Other experimental conditions were the same as those in Figure 2. Open circles showed the average values from three experiments.

by assuming that the two active centers functioned alternately rather than independently, since in the latter case, the activity level should remain at a constant level of 100%. Whether the two active centers of fatty acid synthetase function simultaneously or not has been studied about the enzyme from several sources, namely chicken liver (9), lactating rat mammary gland (10) and rat liver(11). These studies concluded that the two active centers are independently active on the basis that the paired active centers were both occupied by substrates simultaneously. Our results is not contradictory with such results because the observed negative interference may affect individual steps of partial reactions of two loaded centers rather than completing the entire reaction on one active center before the other one is loaded with acetyl-CoA. Experiments to differentiate such possibilities are now progress in our laboratory.

ACKNOWLEDGMENTS

We thank Prof. Yoichi Hashimoto of the Department of Biochemistry, Saitama University, for the generous gift of P. ostreatus protease B, and Dr. Kawaguchi for helpful discussions and comments. This work was supported in part by a Grant-in-Aid from the Ministry of Education of Japan to A.I. (#6222009 and #63580211).

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