Evidence that the multifunctional polypeptides of vertebrate and fungal fatty acid synthases have arisen by independent gene fusion events

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The enoyl reductase (NADPH binding site) of rabbit mammary fatty acid synthase has been radioactively labelled using pyridoxal phosphate and sodium [^{3}H]borohydride. Using this method we have been able to add this site to the four sites whose location has already been mapped within the multifunctional polypeptide chain of the protein. The results show that the enoyl reductase lies between the 3-oxoacylsynthase and the acyl carrier. This confirms that the active sites occur in a different order on the single multifunctional polypeptide of vertebrate fatty acid synthase and the two multifunctional polypeptides of fungal fatty acid synthase, and suggests that these two systems have arisen by independent gene fusion events.

Multifunctional protein *Fatty acid synthase* *Active site labelling* *Enoyl reductase*

1. INTRODUCTION

Although the basic mechanism of fatty acid synthesis from malonyl-CoA is the same in all species, the proteins which catalyze the pathway occur in strikingly different structural organisations [1]. The six catalytic sites and the acyl carrier function are present on separate monofunctional proteins in *Escherichia coli* and plant plastids, on two multifunctional polypeptides in fungi, and on a single multifunctional polypeptide in vertebrates. While limited proteolysis and amino acid sequence data [1,2] now strongly suggest that the multifunctional forms have arisen by fusion of genes coding for monofunctional proteins, it has not been clear whether the vertebrate enzyme represents a fusion of two fungal-type polypeptides, or whether the fungal and vertebrate systems arose by independent gene fusion events. We have recently mapped the locations of four of the active centres of rabbit mammary fatty acid synthase using limited proteolysis [3]. In the present paper we use a previously described method for labelling of the NADPH-binding site of the enoyl reductase [4] to add the location of this function to our map. The results do not support the hypothesis that the fungal and vertebrate enzymes represent sequential stages in evolution, and suggest rather that they arose by independent gene fusion events.

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2. METHODS

Pyridoxal phosphate, 2′-monophospho-ADP ribose, crotonyl-CoA and *S*-acetoacetyl-*N*-acetylcysteamine were from Sigma Chemical Co. (Poole, Dorset). $NaBH_4$ was from BDH Chemicals (Poole, Dorset). NaB^3H_4 was from Amersham International (Amersham, Bucks). Purification of fatty acid synthase, methods for electrophoresis and fluorography, and sources of other materials are as in [3]. Enoyl reductase and 3-oxoacyl reductase activities were assayed using crotonyl-CoA

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(0.5 mM) and *S*-acetoacetyl-*N*-acetylcysteamine (3 mM) as substrates, respectively, as in [5].

Modification of fatty acid synthase by pyridoxal phosphate and $NaBH_4$ was based on [4]. Fatty acid synthase (4 mg/ml) was incubated at 25°C with pyridoxal phosphate (1 mM), with or without 2′-monophospho-ADP ribose (10 mM), in 0.5 M Na-phosphate buffer, 1 mM EDTA (pH 7.0). Where indicated the reaction was stopped by addition of $NaBH_4$ to a final concentration of 5 mM, followed by incubation in ice for 10 min. Before enzyme assays the mixture was then incubated with 0.1% (v/v) 2-mercaptoethanol for 30 min at 37°C. For the experiments in section 3.2, fatty acid synthase was pretreated with elastase as indicated in the legend to fig.2. Proteolysis was stopped by adding diisopropylfluorophosphate to a final concentration of 1 mM, prior to addition of pyridoxal phosphate. In these experiments NaB^3H_4 was used (about 450 Ci/mol) and the labelled protein was precipitated by addition of 10 vol. 25% (w/v) trichloroacetic acid. The precipitate was isolated by centrifugation, washed twice with 1 ml 25% (w/v) trichloroacetic acid, washed once with 1 ml water, and dissolved in 10 μl 90% (w/w) formic acid. For determination of the total radioactivity, these samples could be counted in 1 ml multipurpose scintillation cocktail (Fisons Fine Chemicals, Loughborough). For gel electrophoresis, 200 μl water was added. After lyophilisation the residue was dissolved in sodium dodecyl sulphate sample buffer containing 8 M urea and analysed by electrophoresis as in [3].

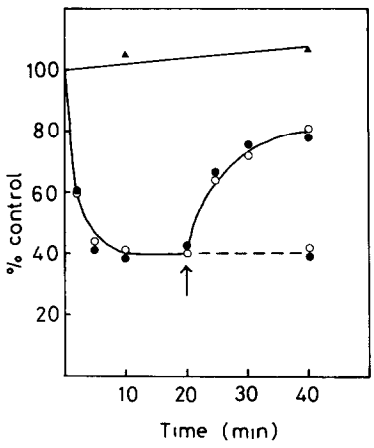

Fig.1. The effect of pyridoxal phosphate on: fatty acid synthase (○); enoyl reductase (●); and 3-oxoacylreductase (▲) activities. The initial incubations were done as in section 2. At the point indicated by the arrow, the enzyme was diluted 200-fold with 0.5 M phosphate, 1 mM EDTA (pH 7.0), in the presence (– – –) or absence (——) of 5 mM $NaBH_4$, and incubated further at 25°C.

The specific activity of the NaB^3H_4 was estimated by adding an aliquot of the NaB^3H_4 solution to excess pyridoxal phosphate. The decrease in absorbance at 388 nm (ϵ = 4900 $l.mol^{-1}.cm^{-1}$, [6]) was measured and the concentration of $NaBH_4$ calculated from this value assuming a stoichiometry of 1 for the reduction. The solution was acidified and evaporated to dryness. The remaining radioactivity, corrected for a blank value in the absence of pyridoxal phosphate, was used to estimate the specific radioactivity of the NaB^3H_4.

3. RESULTS

3.1. *Effect of pyridoxal phosphate on fatty acid synthase activity*

Rabbit mammary fatty acid synthase was inactivated in a time-dependent manner on incubation with pyridoxal phosphate. The final degree of inactivation depended on the concentration used, with 60% inactivation being obtained using 1 mM pyridoxal phosphate (fig.1). The loss in activity was partially recovered on diluting out the pyridoxal phosphate, unless sodium borohydride was added. This behaviour suggests the formation of Schiff's base(s) between pyridoxal phosphate and amino-group(s) on the enzyme, with borohydride reduction preventing the reverse reaction [4]. The enoyl reductase and overall fatty acid synthase were inactivated by pyridoxal phosphate, and reactivated on dilution, with identical kinetics, while 3-oxoacyl reductase activity was unaffected by this treatment (fig.1). The inactivations of enoyl reductase and overall fatty acid synthase activity produced by incubation for 10 min with 1 mM pyridoxal phosphate were reduced by 30% and 32%, respectively, in the presence of 10 mM 2′-monophospho-ADP-ribose. Similar protection was obtained using either NADPH or $NADP^+$ (not shown).

Using excess NaB^3H_4 to reduce the Schiff's bases, stoichiometries of 3.0 and 1.8 pyridoxal

phosphate moieties incorporated per subunit were obtained in the absence and presence respectively of 10 mM 2′-monophospho-ADP-ribose. Due to inherent inaccuracy in the measurement of specific radioactivity of [^{3}H]borohydride, these values should be regarded as approximate.

3.2. *Mapping of the enoyl reductase site by limited proteolysis*

We have already mapped the positions of four of the active centres of rabbit mammary fatty acid synthase on five fragments of the polypeptide chain which have been generated and ordered using limited elastase digestion [3]. We have now applied the same procedure to the mapping of the enoyl reductase site. Fig.2A shows the results of limited digestion of pyridoxal phosphate/NaB^{3}H$_4$-labelled fatty acid synthase using two different preparations of elastase. Using the best preparation of elastase (fig.2A; 3,4) radioactivity is located in all of the fragments, but the addition of the NADPH analogue 2′-monophospho-ADP-ribose results in a selective decrease in labelling of F120.

This is clearly seen in densitometric scans of the fluorogram (fig.2B). In three different experiments the radioactivity in F120 was decreased by 33 ± 5% (mean ± standard error of the mean) relative to the radioactivity in F110. Under the same conditions the analogue provides 41 ± 9% protection of fatty acid synthase activity against inactivation by pyridoxal phosphate. Radioactivity in F220 is also decreased slightly, which is consistent with our proposal that F120 is derived from F220 [3]. The thioesterase fragment (F35 [3]) is not visible in fig.2 due to the conditions used for electrophoresis. Separate experiments showed that while this fragment did become radioactive, the labelling was not affected by 2′-monophospho-ADP-ribose (not shown).

We also used another batch of elastase which produces a slightly different cleavage pattern, probably because it is contaminated with traces of other proteases [3]. In this case, the fragments are not resolved well enough to allow densitometry, but it can be clearly seen by inspection of the fluorogram (fig.2A; 1,2) that the NADPH analogue reduces the labelling of F102 and F93, while not affecting the labelling of F110. F102 and F93 are derived by removal of small fragments from the ends of F120 [3].

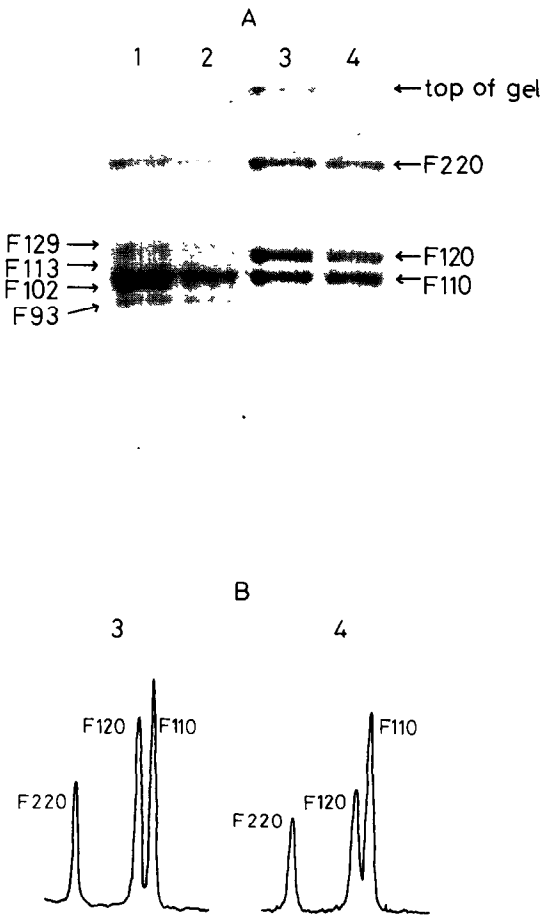

Fig.2. The effect of 2′-monophospho-ADP-ribose on labelling of fatty acid synthase proteolytic fragments by pyridoxal phosphate/NaB^{3}H$_4$. (A) Fluorograms of fragments labelled in the presence (2 and 4) or absence (1 and 3) of 10 mM 2′-monophospho-ADP-ribose. The enzyme was digested for 20 min at 25°C at a substrate/protease ratio of 125:1 using elastase from Boehringer Mannheim GmbH (lot 139 1411) (tracks 3,4) or Sigma Chemical Co. (lot 100F-8075) (tracks 1,2). The numbering of the fragments is taken from [3]. (B) Densitometric scans of tracks 3 and 4.

4. DISCUSSION

Although it is clear from the stoichiometry of labelling and from fig.1 that a certain amount of non-specific labelling of rabbit fatty acid synthase occurs using pyridoxal phosphate and NaB^{3}H$_4$ several observations suggest that protection by 2′-monophospho-ADP-ribose provides a specific marker for the nucleotide-binding site of the enoyl

reductase. Poulose and Kolattukudy [4] have shown that of the seven partial reactions of goose fatty acid synthase, only the enoyl reductase is inhibited by treatment with pyridoxal phosphate and sodium borohydride. Protection against inactivation is provided by NADPH and 2′-monophospho-ADP-ribose, but not by adenine nucleotides which lack 2′- or 5′-phosphate groups. They have also isolated a peptide which is protected by 2′-monophospho-ADP-ribose and have shown convincing sequence homology with the nucleotide-binding domains of several dehydrogenases [7]. We have confirmed that pyridoxal phosphate/sodium borohydride treatment of rabbit fatty acid synthase results in parallel inactivation of overall fatty acid synthase and enoyl reductase activity, without affecting 3-oxoacyl reductase activity. Substantial protection against inactivation can be obtained using either NADPH, $NADP^+$ or 2′-monophospho-ADP-ribose. We have used the last compound in our radioactive labelling experiments because the first two compounds interfere with the borohydride reduction.

The present results demonstrate that the nucleotide-binding site of rabbit fatty acid synthase enoyl reductase is present on elastase fragments F120, F102, and F93, and in combination with [3], allow us to produce a more detailed map of the single polypeptide chain of this multifunctional complex. In fig.3, this map is compared with the disposition of the active sites between the two subunits of yeast fatty acid synthase. The enoyl reductase lies between the 3-oxoacylsynthase and the acyl carrier in the rabbit subunit, yet in yeast the enoyl reductase is on the β-subunit while the 3-oxoacylsynthase and acyl carrier are on the α-subunit.

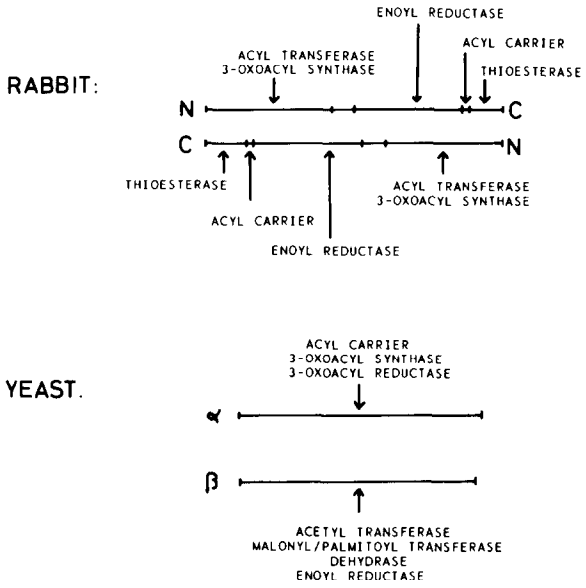

Fig.3. Comparison of the distribution of active centres on the single polypeptide chain of rabbit fatty acid synthase and the two subunits of the yeast enzyme. The rabbit enzyme is shown as a dimer in 'head-to-tail' configuration as suggested in [12]. Data from the yeast enzyme are from [13].

It is not therefore possible to generate a rabbit-type polypeptide by fusion of two yeast-type polypeptides or, conversely, to generate two yeast-type polypeptides by cleavage of a rabbit-type subunit. There are several other intriguing differences between the rabbit and yeast systems.

(1) The yeast system has separate acetyl and malonyl transferases, while a single site catalyses both functions in the rabbit enzyme [8].

(2) The yeast system terminates fatty acid synthesis by transfer of the fatty acid to coenzyme A, while the rabbit enzyme uses hydrolysis by the thioesterase [1].

(3) The yeast enoyl reductase contains bound flavin mononucleotide, unlike the rabbit enzyme [9].

(4) The two yeast subunits contain about 50% more polypeptide than the single rabbit subunit.

(5) The native yeast enzyme contains six copies of each subunit [1]; whereas the rabbit enzyme is a dimer [11].

We therefore propose that the fungal and vertebrate fatty acid synthase systems do not represent sequential stages in evolution, but rather that they arose by gene fusion events which were, at least in part, independent.

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