LOCALIZATION OF THREE NON-THIOL BINDING SITES ON POLYPEPTIDE CHAIN β OF YEAST FATTY ACID SYNTHETASE

Hansjörg ENGESER, Felix WIELAND and Feodor LYNEN
Max-Planck-Institut für Biochemie, D-8033 Martinsried, FRG

Received 21 July 1977

1. Introduction

The multienzyme complex FAS from yeast is known to consist of two different multifunctional polypeptide chains, α and β [1] bearing seven different partial enzymic activities [2]. The distribution of six partial enzymes on the subunits has so far only been proposed from genetic studies [1]. A localization of the partial enzyme acetyl transferase until now has not been achieved. The genetic prediction for the localization of the integrated ACP as well as the condensing activity has been proven by protein chemical methods [1,3].

SDS–gel electrophoresis, in our hands, did not yield clear separation of yeast FAS polypeptide chains. Furthermore, the long duration of this method allows the possibility of cleavage of labile chemical bonds between active sites and radioactively labeled markers used for such localization studies.

Therefore a rapid, high-resolution, gel electrophoresis system has been developed for the localization of non-thiol binding sites (transferases) for acetyl, malonyl, and palmitoyl residues on fatty acid synthetase subunits.

Abbreviations: FAS, fatty acid synthetase, ACP, acyl carrier protein; SDS, sodium dodecylsulfate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); bis, *N,N′*-methylenebisacrylamide; Temed, *N,N,N′,N′*-tetramethylethylenediamine; TEA, triethanolamine, GuHCl, guanidine hydrochloride; CA, citraconic anhydride; THF, tetrahydrofurane

2. Material and methods

2.1. *Commercial materials*

Citraconic anhydride, DTNB were from Fluka, Bucks. CoA, NADPH were from Boehringer, Mannheim. Acrylamide, bis, Temed and Coomassie Brilliant Blue G-250 were from Serva, Heidelberg. TEA/HCl and 2-mercaptoethanol were from Roth, Karlsruhe. Iodo-[1-^{14}C]acetamide, Na$H^{14}CO_3$ were obtained from the Radiochemical Centre, Amersham. [1-^{14}C]Palmitoyl-CoA was purchased from NEN, Dreieichenhain.

All other chemicals used were from Merck, Darmstadt, in the highest purity grade available. Iodoacetic acid was recrystallized from a pentane/heptane (1:1, v:v) mixture. Dimilume 30 and soluene 350 were from Packard Instrument, Frankfurt.

Fatty acid synthetase was isolated according to Lynen [4] and exhibited specific activities between 2000 and 3000 mU/mg. (1 mU = 2 nmol NADPH oxidized/min at 25°C).

Acetyl-CoA-carboxylase from yeast was a gift of Dr J. Spiess. Protein was determined by the biuret method [5] or spectrophotometrically at 280 nm. 1 mg protein/ml yields an absorbancy of 1.15 (d = 1 cm).

2.2. *Substrates*

Acetyl-CoA was prepared according to Simon and Shemin [6]. [1-^{14}C]Acetyl-CoA was a gift of Dr R. Seyffert. Malonyl-CoA was prepared according to Eggerer and Lynen [7]. [3-^{14}C]Malonyl-CoA was prepared enzymatically with yeast acetyl-CoA-carboxylase and purified according to Higgins and Kekwick [8].

2.3. *Methods*

FAS for all localization studies was treated as follows: A solution of FAS (20 mg/ml) in 0.1 M KPO_4 buffer, pH 6.5, was incubated with 10^{-3} M DTNB at room temperature for 2 h.

2.3.1. [1-^{14}C]Acetyl-CoA incubation

A sample containing 10 mg/ml enzyme was then incubated at 0°C for 8 min with 10^{-3} M [1-^{14}C]acetyl CoA (spec. radioact. 44.1 mCi/mmol). The reaction was stopped by pouring the mixture into 2 vol. denaturation solution: 7 M GuHCl, 0.7 M TEA/HCl, 2.5×10^{-3} M DTNB brought to pH 8.2 with NaOH. The samples were kept for 15 min at room temperature prior to citraconylation.

2.3.2. [3-^{14}C]Malonyl-CoA incubation

The DTNB-treated enzyme (20.3 mg/ml) was incubated with 1.1×10^{-4} M [3-^{14}C]malonyl-CoA (29 mCi/mmol) for 30 min at 0°C and denatured as described above.

2.3.3. [1-^{14}C]Palmitoyl-CoA incubation

The DTNB-treated enzyme (13 mg/ml) was allowed to stand at 0°C in the presence of 7×10^{-5} M [1-^{14}C]palmitoyl-CoA (spec. radioact. 60 mCi/mmol) for 15 min and denaturated as described above.

2.3.4. Labeling of the α subunit

Labeling of the α subunit with iodo-[1-^{14}C]acetamide was performed according to Kresze et al. [9]. [1-^{14}C]Carboxamidomethyl fatty acid synthetase (9 mg/ml) was treated with DTNB and denatured as described in sections 2.3.1.–3.

2.4. *Citraconic anhydride treatment*

To 600 μl of incubations 2.3.1.–4., 60 μl of a 1:1 (v:v) mixture of CA/THF (freshly prepared) were added in 10 portions within 10 min at 0°C under shaking. The pH of the solution was maintained at about 8.2 (glass electrode) by subsequent addition of 9–11 60 μl portions of 1 N NaOH. Thereafter the solutions were allowed to react for another 20 min.

Reduction and carboxymethylation were performed essentially according to Crestfield et al. [10]. The pH was raised to 8.4 and then to 900 μl aliquots of the citraconylated samples, 8 μl of 2-mercaptoethanol were added. After 30 min at 40°C 75 μl of a 0.268 g iodoacetic acid in 1 ml 1 N NaOH (freshly prepared) solution were added and allowed to react for 15 min at room temperature, the pH being kept above 8.0 by addition of small amounts of NaOH.

Dialysis against 500 vol. Maurer system 1 electrophoresis buffer [11] containing 6 M urea was performed under stirring for about 2 h at room temperature.

The dialysed samples were subjected to electrophoresis on gels containing 3.4% acrylamide, 0.1% bis, 0.029% Temed and 6 M urea. Electrophoresis system: Maurer I [11]. Running time: 2 h at 4°C and 250 V (stabilized).

The gels were stained with Coomassie Brilliant Blue G-250 (0.25% in methanol/acetic acid/H_2O, 10:7:83) for 1 h at room temperature and destained overnight in methanol/acetic acid/H_2O, 10:7:83. The destained gels were frozen in acetone/dry ice and sliced into 14–20 equal fractions which were incubated with 1 ml soluene 350 and 100 μl H_2O for 2 h at 40°C.

Dimilume 30, 10 ml, were added and the samples were counted in a Mark II liquid scintillation counter (Nuclear Chicago).

3. Results

Gel electrophoresis of the carboxymethylated and citraconylated samples yielded 2 sharp protein bands 1.5 mm apart. A third faint band sometimes was visible in front of the faster main zone which could be verified by microscopic magnification (fig.1.).

To exclude that the described chemical modification leads to subunit mixtures modified to a different extent, FAS was labeled with the α subunit-specific reagent iodo-[1-^{14}C]acetamide and subjected to the modification and separation procedure.

The analysis of radioactivity in the fractionated gels showed incorporation of the [^{14}C]carboxamidomethyl residues exclusively into the faster moving zone (band II, fig.2).

This clearly indicates a separation of FAS into 2 essentially homogeneous subunits. Since band II resembles α subunit [4,9], it follows that zone I must be β subunit. This separation suggested the possibility of a protein–chemical localization of binding sites for covalently-linked substrates. Therefore active thiol groups like the active sites of the

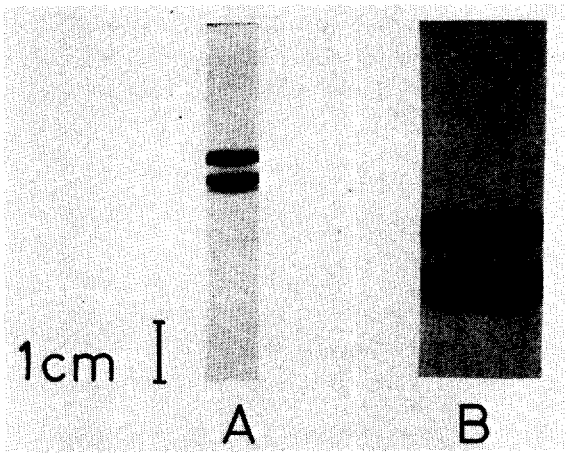

Fig.1. Separation of FAS polypeptide chains. FAS (6 mg/ml) was treated with DTNB, citraconic anhydride, 2-mercaptoethanol and iodoacetic acid was described in Materials and methods. Dialyzed samples, 10 μg, were applied to gels (0.4 × 6 cm) and run as described in the Methods section. (A) Photograph of the gel. (B) Magnified 2.3 times.

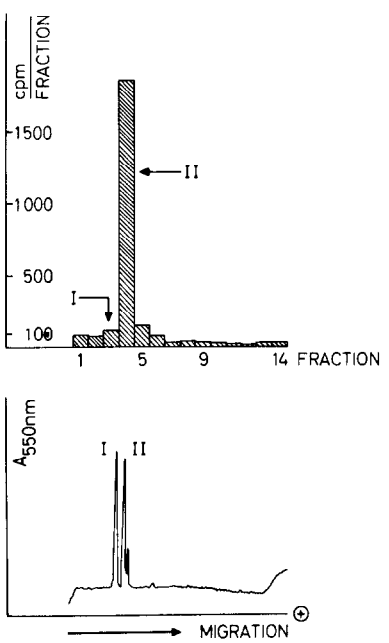

Fig.2. Localization of FAS α subunit. FAS was treated with iodo-[1-^{14}C]acetamide, a specific label for α subunit [4,9] and modified and run on gels as described in the Methods section. (A) Distribution of radioactivity. (B) Densitometric scanning profile of the gel after staining (at 550 nm).

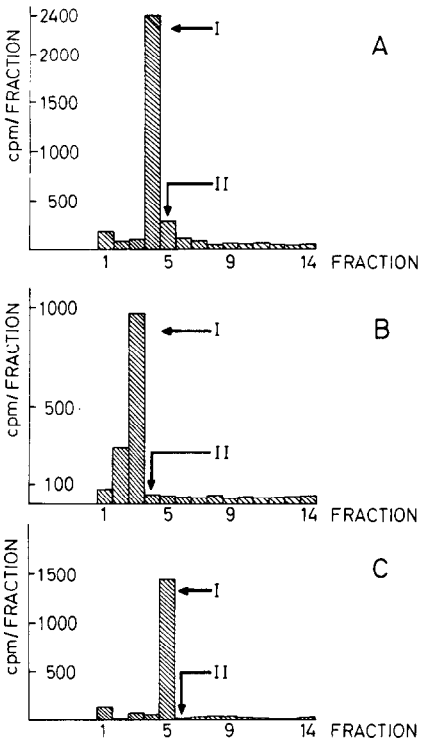

Fig.3. Localization of FAS acetyl-, malonyl-, and palmitoyl-transferases on β subunit. FAS was incubated with [1-^{14}C]-acetyl-, [3-^{14}C]malonyl- and [1-^{14}C]palmitoyl-CoA and treated as described in the Methods section. Distribution of radioactivity of (A) [1-^{14}C]acetyl-FAS, (B) [3-^{14}C]malonyl-FAS, (C) [1-^{14}C]palmitoyl-FAS.

integrated acyl carrier protein (–ScH) and of the condensing enzyme (–SpH) were blocked with DTNB, and the non-thiol active residues of the transferases were specifically labeled with the radioactive substrates [^{14}C]acetate, [^{14}C]malonate and [^{14}C]palmitate. The radioactivity introduced by specific binding of these substrates in all cases was found to be incorporated into the protein band I, namely β subunit (fig.3,A,B,C).

4. Discussion

Previously reported results [3,9] described an absolute specificity in the reaction of iodoacetamide with yeast FAS for the α subunit (condensing enzyme).

Finding this marker exclusively in one protein band we were quite confident that a separation into two different subunits had been achieved, and that the slower moving protein resembled β subunit. In agreement with this result was the observation of a splitting of the α band into a main zone and a further faster-migrating faint band which was observed by Schweizer et al. [1] on SDS gels of wild-type yeast FAS and explained by proteolytic degradation of the α subunit. In the system described, migration was obviously not only determined by the molecular weight of the proteins but also by their charge which was changed in net by 2 negative charges per one ϵ-amino group of lysine citraconylated. In agreement with this migration behaviour was the preferential occurrence of Glx and Lys residues in the polypeptide α chain as determined by amino acid analysis of the isolated polypeptide chains (Wieland, F., in preparation).

As previously reported [12], blocking of active thiol groups with DTNB permits specific loading of the transferases and has been described in detail for acetyl transferase by Ziegenhorn et al. [13]. The short separation time of the method described allowed the localization of the three enzymes, acetyl, malonyl and palmitoyl transferase of yeast FAS on β subunit, in spite of the relative lability of the enzyme substrate oxygen ester-bonds. The analytical evaluation of the subunit separation was facilitated by the high resolution power of this procedure, the more general applicability of which will be described elsewhere.

The result of this protein–chemical localization study concerning malonyl- and palmitoyl-transferases is in agreement with the proposal of Knobling et al. [14] based on genetic studies. Furthermore it was possible to assign acetyl transferase also to polypeptide β chain.

The binding of malonyl and palmitoyl binding sites on the same polypeptide chain is in good agreement with other observations indicating an identity of malonyl transferase and palmitoyl transferase [12,14–16].

References

[1] Schweizer, E., Kniep, B., Castorph, H. and Holzner, U. (1973) Eur. J. Biochem. 39, 353–362.
[2] Lynen, F. (1961) Fed. Proc. Fed. Am. Soc. Exp. Biol. 20, 941–951.
[3] Kresze, G. B., Oesterhelt, D., Lynen, F., Castorph, H. and Schweizer, E. (1976) Biochem. Biophys. Res. Commun. 69, 893–899.
[4] Lynen, F. (1969) in: Methods in Enzymology (Lowenstein, J. M. ed) vol. 14, pp. 17–33, Academic Press, New York.
[5] Beisenherz, G., Boltze, H. J., Bücher, Th., Czok, R., Garbade, K. H., Meyer-Arendt, E. and Pfleiderer, G. (1953) Zeitschr. f. Naturforsch. 8b, 555–577.
[6] Simon, E. J. and Shemin, D. (1953) J. Amer. Chem. Soc. 75, 2520.
[7] Eggerer, H. and Lynen, F. (1962) Biochem. Z. 335, 540–547.
[8] Higgins, M. J. P. and Kekwick, R. G. O. (1973) Biochem. J. 134, 295–310.
[9] Kresze, G. B., Steber, L., Oesterhelt, D. and Lynen, F. (1977) Eur. J. Biochem. in press.
[10] Crestfield, A. M., Moore, S. and Stein, W. H. (1963) J. Biol. Chem. 238, 622.
[11] Maurer, H. R. (1971) in: Disc-Electrophoresis, Walter de Gruyter, Berlin, New York.
[12] Engeser, H., Hirsch, A. and Lynen, F. (1976) 10th Int. Congr. Biochem. Hamburg, Abstr. 04-3-333.
[13] Ziegenhorn, J., Niedermeier, R., Nüssler, C. and Lynen, F. (1972) Eur. J. Biochem. 30, 285–300.
[14] Knobling, A., Schiffmann, D., Sickinger, H.-D. and Schweizer, E. (1975) Eur. J. Biochem. 56, 359–367.
[15] Ayling, J., Pirson, R. and Lynen, F. (1972) Biochemistry 11, 526–532.
[16] Schreckenbach, Th. (1975) Ph. D. thesis, University of Munich.