Biochem. J. (1991) 280, 353–357 (Printed in Great Britain)

Identification of the CoA-modified forms of mitochondrial acetyl-CoA acetyltransferase and of glutamate dehydrogenase as nearest-neighbour proteins

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A 52 kDa protein could only be co-purified with the CoA-modified forms of acetyl-CoA acetyltransferase (acetoacetyl-CoA thiolase) (EC 2.3.1.9) from rat liver mitochondria. Immunoprecipitations of these modified forms with anti-(acetyl-CoA acetyltransferase) IgG or anti-(52 kDa protein) IgG yielded, in addition to the appropriate proteins, the 52 kDa protein or the CoA-modified form of acetyl-CoA acetyltransferase (41 kDa) respectively. This was demonstrated by SDS/PAGE and immunoblots. The modified forms containing the 52 kDa protein could be cross-linked by 1,5-difluoro-2,4-dinitrobenzene to a high-molecular-mass complex containing both the 41 kDa and 52 kDa proteins. The 52 kDa protein was identified as mitochondrial glutamate dehydrogenase (EC 1.4.1.3) by amino acid sequence analysis. The results of co-immunoprecipitation and cross-linking characterize the CoA-modified forms of acetyl-CoA acetyltransferase and the glutamate dehydrogenase as nearest-neighbour proteins.

INTRODUCTION

Among the mitochondrial proteins showing [^{14}C]pantothenic acid-derived radioactivity, two proteins of *Neurospora crassa* have been shown to be covalently modified by 4′-phosphopantotheine [1]. By means of administration of [1-^{14}C]pantothenic acid *in vivo* to rats, the liver mitochondrial matrix enzymes acetyl-CoA acetyltransferase (acetoacetyl-CoA thiolase) (EC 2.3.1.9) and the 3-oxoacyl-CoA thiolase (EC 2.3.1.16) were identified as radioactively labelled proteins [2]. Further, it has been demonstrated that the observed charge heterogeneity of these proteins and the incorporated radioactivity [2] are caused by a specific modification with CoA [3]. The CoA-binding showed a thiol-instability, suggesting a covalent attachment of CoA to the proteins as a mixed disulphide. This post-translational CoA modification of, for example, the acetyl-CoA acetyltransferase protein results in the forms A2 and A1, which reveal only partial catalytic activity [4] and, corresponding to the modification, exhibit continually increasing specific radioactivities in a kinetic experiment after pulse-labelling with [1-^{14}C]pantothenic acid [2]. In a different approach pulse-labelling with [^{14}C]leucine resulted in a significantly higher specific radioactivity of the modified forms when compared with the unmodified enzyme [2]. These data suggest a time-delaying interaction of the modified forms, presumably with other mitochondrial-matrix proteins. Thus it is highly interesting that the CoA modification of these forms is transient, i.e. the fully active acetyl-CoA acetyltransferase does not show the modification by CoA [3]. The acetyl-CoA acetyltransferase is encoded in the nucleus, synthesized as a precursor, transported into the matrix of mitochondria and then processed to its mature size [5]. In the matrix, the imported proteins are folded and assembled to become functionally active under the participation of heat-shock proteins [6,7].

In the present paper we describe the co-purification of the CoA-modified forms of the mitochondrial-matrix enzyme acetyl-CoA acetyltransferase with an additional protein. Both of these proteins could be co-immunoprecipitated and could be cross-linked by 1,5-difluoro-2,4-dinitrobenzene, the reagent of choice for the analysis of nearest-neighbour proteins [8]. The co-purified unknown protein was identified as the mitochondrial-matrix enzyme glutamate dehydrogenase.

MATERIALS AND METHODS

Materials

Matrex Gel Red A was from Amicon G.m.b.H. (Witten, Germany). Sephacryl S-200 (superfine grade), Protein A–Sepharose, Ultrogel AcA 34 and the calibration proteins for gel filtration were from Pharmacia–LKB (Freiburg, Germany). Phosphocellulose, DEAE-cellulose and nitrocellulose sheets were from Schleicher und Schüll (Dassel, Germany). Immobilon-P membranes were from Millipore G.m.b.H. (Eichborn, Germany). Calibration proteins for SDS/PAGE were from Sigma (Deisenhofen, Germany). *Staphylococcus aureus* V8 proteinase was from Boehringer–Mannheim (Mannheim, Germany). 1,5-Difluoro-2,4-dinitrobenzene was from Pierce (Köln, Germany) and di-(2-ethylhexyl) phthalate was from EGA-Chemie (Steinheim, Germany). All other chemicals were of high purity and were obtained from common commercial sources. Acetoacetyl-CoA was prepared, purified and assayed as described in ref. [9].

Purification of acetyl-CoA acetyltransferase

Five male Wistar rats (150–180 g) were fed with a standard diet containing 2% (w/v) di-(2-ethylhexyl) phthalate, known as an inducer of acetyl-CoA acetyltransferase [5], for 6 days. Liver mitochondria and the mitochondrial extract were prepared as described in ref. [2] and solubilized in a medium of 50 mM-potassium phosphate buffer, pH 8.0, containing 1 mM-EDTA, 1 mM-GSH, 1 mM-phenylmethanesulphonyl fluoride and 20% (w/v) glycerol. This medium was used in all chromatographic steps; the pH value and the K^+ concentration were changed as indicated. The activity of acetyl-CoA acetyltransferase was assayed for acetoacetyl-CoA cleavage as described in ref. [10].

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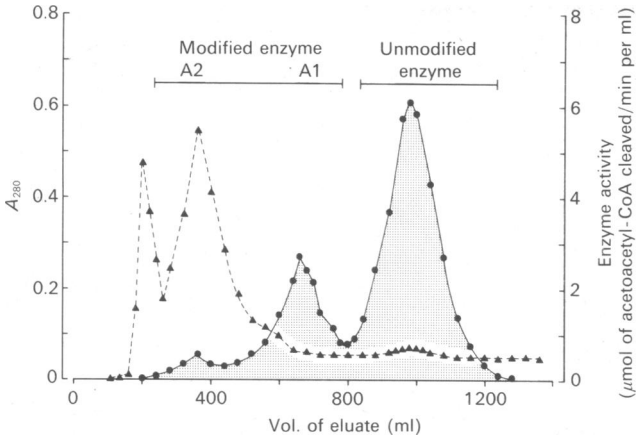

Fig. 1. Resolution of acetyl-CoA acetyltransferase activity into three peaks by chromotography on phosphocellulose

●, Enzyme activity; ▲, A_{280}. A 2046 mg portion of protein was transferred to a column (2.5 cm × 45 cm), equilibrated with the medium (pH 6.8, 0.2 M-K^+) and eluted by a gradient of 0.25–0.75 M-K^+. As indicated by the horizontal lines the activity was pooled for the modified forms A2/A1 and the unmodified enzyme. The modified forms show a specific activity of 3.0 units/mg of protein.

Protein concentration was measured by the method of Lowry *et al.* [11], with BSA as standard.

The mitochondrial extract was chromatographed on DEAE-cellulose, whereby the acetyl-CoA acetyltransferase activity passes through the column directly. Then the protein solution (2046 mg of protein) was adjusted to pH 6.8, and subjected to cation-exchange and gel-filtration chromatography (see Figs. 1 and 2).

PAGE

SDS/PAGE was performed according to the procedure of Laemmli [12] with 12% (w/v) polyacrylamide. Calibration proteins were phosphorylase *b* (97 kDa), BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa) and trypsin inhibitor (20.1 kDa). For gradient SDS/PAGE, 3% (w/v) polyacrylamide was used in the stacking gel and a 3–22% (w/v) polyacrylamide gradient in the separation gel. Calibration proteins were myosin (206 kDa) phosphorylase *b* (97 kDa) and BSA (66 kDa). The proteins were stained with Coomassie Brilliant Blue.

Immunochemical procedures

Preparation of antibodies. Antibodies against the liver mitochondrial acetyl-CoA acetyltransferase and glutamate dehydrogenase (52 kDa protein) were raised in rabbits and the IgG fraction was isolated from each antiserum as described in ref. [13].

Immunoprecipitation. An excess of antibodies (120 μg of IgG/μg of immunospecific protein) was added to a reaction mixture (100 μl) containing 5 μg of immunospecific protein in 100 mM-NaCl/20 mM-potassium phosphate buffer, pH 7.2. After incubation for 60 min at 37 °C and overnight at 4 °C, immunoprecipitates were collected by centrifugation (15000 *g* for 15 min), washed and solubilized in suitable buffer for SDS/PAGE.

Immunoblot. The discontinuous system for semi-dry blotting according to the procedure of Kyhse-Anderson [14] was used when the enzyme or its immunoprecipitates were electroblotted on to nitrocellulose sheets. The cross-linked high-molecular-complexes were immunoblotted on nitrocellulose sheets as described in refs. [15] and [2] for 15 h at 12 V (constant) and for 3 h at 200 mA (constant). Bound proteins were stained with Ponceau S.

Cross-linking experiments

For cross-linking the proteins were incubated for 1.5 h at 37 °C in a reaction mixture containing 1 mg of protein/ml in 1 mM-1,5-difluoro-2,4-dinitrobenzene in 25 mM-Tris/HCl buffer, pH 8.8. The cross-linked proteins were analysed by gradient SDS/PAGE and immunoblots.

Peptide mapping and sequencing

Following the SDS/PAGE of the modified forms A2/A1, the 52 kDa protein was excised and transferred (12 μg) to a second SDS/PAGE gel, overlayed with a buffer (Tris/HCl, pH 6.8) and a V8 proteinase (750 ng)-containing buffer as described in ref. [16]. After this SDS/PAGE the suitable lane of the gel was excised and the proteins were transferred on to Immobilon-P transfer membranes by means of the above-mentioned semi-dry system of electroblotting. The peptides were stained with Coomassie Brilliant Blue. The appropriate peptide was excised and analysed with an Applied Biosystems gas-phase sequencer [17] with an on-line h.p.l.c. system [18].

RESULTS

Apparent co-purification of the CoA-modified forms of acetyl-CoA acetyltransferase with a 52 kDa protein

During the purification of acetyl-CoA acetyltransferase by suitable chromatographic steps, the enzyme protein was separated into the unmodified enzyme and its CoA-modified forms A2 and A1 by cation-exchange chromatography (Fig. 1) as previously described [2]. According to the elution profile of the enzymic activity the modified forms and the unmodified enzyme amounted to 30.6% and 69.4% of total activity respectively. The pooled modified forms A2/A1 appeared in an Ultrogel AcA34 gel filtration in two activity peaks with molecular masses of 460 kDa and 250 kDa within one protein peak (result not shown). Then for a separation of these activity peaks we used Sephacryl S-200 columns. Fig. 2(*a*) demonstrates that the activity pattern persisted: the first activity peak resembles the 460 kDa peak of the Ultrogel AcA34 column and is, as expected, out of the fractionation range. The second peak reveals a molecular mass of 250 kDa. However, when this column sizing was repeated under identical conditions but with the pH elevated from 6.8 to 7.4, one symmetrical activity peak with a molecular mass of 151 kDa was obtained (Fig. 2*b*) and most of the contaminating proteins, e.g. a 72 kDa protein, became separated (Fig. 3, lanes A and B). From the SDS/PAGE (Fig. 3, lane B) it is also evident that in addition to the 41 kDa enzyme protein a 52 kDa protein is contained in this activity peak in a ratio of 35.4% to 64.6% (densitometric scanning). The immunoblot with anti-(acetyl-CoA acetyltransferase) IgG identifies only the 41 kDa protein (Fig. 3, lane D). By contrast, the unmodified enzyme appeared subsequent to gel filtration on Ultrogel AcA34 (result not shown) as a homogeneous immunoreactive protein (Fig. 3, lanes C and E).

From these results it became obvious that a 52 kDa mitochondrial protein has been co-purified with the CoA-modified forms A2/A1 of acetyl-CoA acetyltransferase. To separate these proteins we performed affinity chromatography on Matrex Gel Red A, known for its affinity to acyl-CoA-utilizing enzymes [19]. Obviously, the CoA-modified forms show no affinity to the Matrex Gel Red A, as the main portion of the modified forms (76% of total activity) passed through the

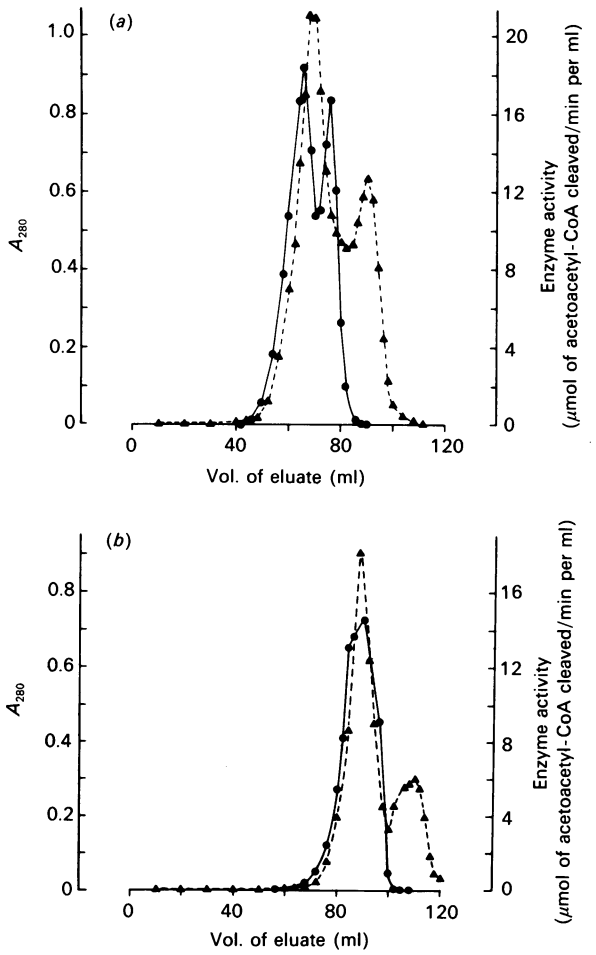

Fig. 2. Gel filtration of the CoA-modified forms A2/A1 of acetyl-CoA acetyltransferase

(*a*) Sephacryl S-200 (superfine grade): ●, enzyme activity; ▲, A_{280}. A 39.3 mg portion of protein was transferred to a column (1.5 cm × 90 cm) equilibrated with the medium (pH 6.8, 0.08 M-K^+). The fractions containing acetyl-CoA acetyltransferase activity were pooled and determined to have a specific activity of 17.9 units/mg of protein. Calibration proteins: catalase (232 kDa), alcohol dehydrogenase (150 kDa), BSA (66 kDa) and ovalbumin (45 kDa). (*b*) Sephacryl S-200 (superfine grade): ●, enzyme activity; ▲, A_{280}. A 19.5 mg portion of protein was transferred to a column equilibrated with the medium (pH 7.4, 0.09 M-K^+). The eluate from 70 ml to 96 ml was pooled and the acetyl-CoA acetyltransferase was determined to have a specific activity of 18.9 units/mg of protein.

column directly and thereby became separated from the 52 kDa protein. However, a definite amount of the modified forms (24 % of total activity) was again co-eluted with the 52 kDa protein by an Na^+ gradient (result not shown). Thus the question arises whether physical interactions of the 52 kDa protein with the modified forms A2/A1 of acetyl-CoA acetyltransferase could exist.

Demonstration of strong physical interactions of 41 kDa and 52 kDa mitochondrial proteins

To clarify whether the 52 kDa protein and the 41 kDa protein of the CoA-modified forms A2/A1 of acetyl-CoA acetyltransferase exert physical interaction we performed immunoprecipitation experiments. Thus, if interactions exist, an antibody against acetyl-CoA acetyltransferase that shows no cross-reactivity with the 52 kDa protein (see Fig. 3, lane D) should co-immunoprecipitate the 52 kDa protein and vice versa.

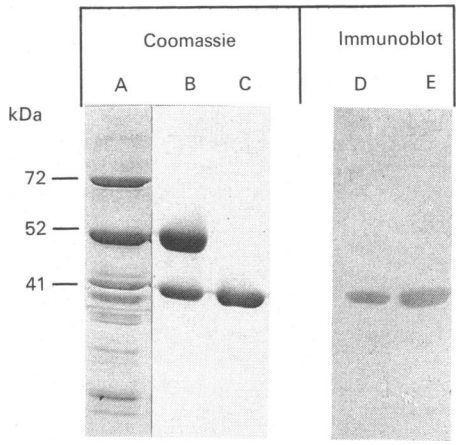

Fig. 3. Co-purification of a 52 kDa protein with the CoA-modified forms of acetyl-CoA acetyltransferase: demonstration by SDS/PAGE and immunoblots

SDS/PAGE: lane A, modified forms following gel filtration as demonstrated in Fig. 2(*a*); lane B, modified forms following gel filtration as demonstrated in Fig. 2(*b*); lane C, unmodified enzyme following gel filtration. Immunoblots with anti-(acetyl-CoA acetyltransferase) IgG: lane D, modified forms following gel filtration as demonstrated in Fig. 2(*b*); lane E, unmodified enzyme following gel filtration.

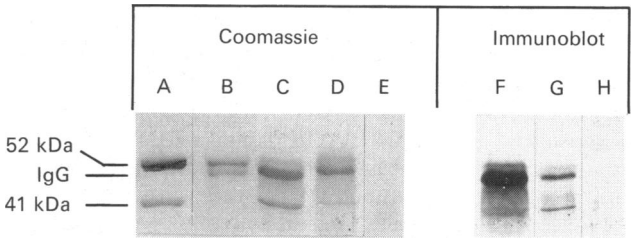

Fig. 4. Immunoprecipitation of the CoA-modified forms A2/A1 of acetyl-CoA acetyltransferase

If not stated otherwise the modified forms A2/A1 (5 μg) were used for these experiments. The proteins or the immunoprecipitates were submitted to SDS/PAGE and analysed by Coomassie Brilliant Blue staining (lanes A–E) and by immunoblots (lanes F–H). Lane A, forms A2/A1; lane B, supernatant of immunoprecipitation with anti-(acetyl-CoA acetyltransferase) IgG; lane C, immunoprecipitate of anti-(acetyl-CoA acetyltransferase) IgG; lane D, immunoprecipitate of anti-(52 kDa protein) IgG; lane E, immunoprecipitation of the unmodified enzyme with anti-(52 kDa protein) IgG; lane F, immunoprecipitate of lane C blotted with anti-(52 kDa protein) IgG; lane G, immunoprecipitate of lane D blotted with anti-(acetyl-CoA acetyltransferase) IgG; lane H, immunoprecipitate of lane E blotted with anti-(52 kDa protein) IgG.

Following application of anti-(acetyl-CoA acetyltransferase) IgG, both the 41 kDa and 52 kDa proteins appeared in the precipitate (Fig. 4, lane C). Although the 41 kDa protein was totally precipitated (lanes A, B and C), some of the 52 kDa protein persisted in the supernatant of the immunoprecipitate (lane B). Additionally, the immunoprecipitated 52 kDa protein was identified by an immunoblot with anti-(52 kDa protein) IgG (lane F). This antibody apparently shows a minor cross-reactivity with the 41 kDa protein. The immunoprecipitation of the forms A2/A1 with anti-(52 kDa protein) IgG resulted in a co-immunoprecipitation of the 41 kDa protein (lane D), which is further confirmed by an immunoblot with the appropriate antibody (lane G). In order to evaluate whether the co-immunoprecipitation of the 41 kDa protein might be due to the

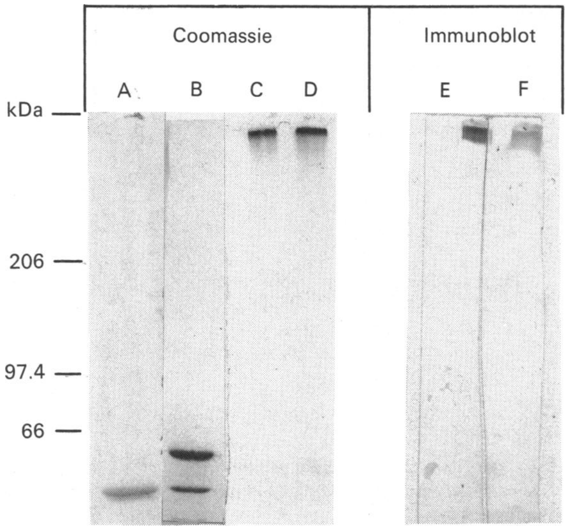

Fig. 5. Cross-linking of the CoA-modified forms A2/A1 of acetyl-CoA acetyltransferase by 1,5-difluoro-2,4-dinitrobenzene: demonstration by gradient SDS/PAGE and immunoblots

Lane A, unmodified enzyme; lane B, modified forms, without 1,5-difluoro-2,4-dinitrobenzene; lanes C and D, modified forms; lanes E and F, immunoblots of the cross-linked modified forms with anti-(acetyl-CoA acetyltransferase) IgG (lane E) and with anti-(52 kDa protein) IgG (lane F).

observed cross-reactivity of the 52 kDa antibody, we performed immunoprecipitation with the unmodified acetyl-CoA acetyltransferase, which lacks the 52 kDa protein. As demonstrated by SDS/PAGE (lane E), there is no immunoprecipitate that could be identified by an immunoblot with the appropriate antibody (lane H).

To ascertain a physical interaction of the modified forms A2/A1 (41 kDa) with the 52 kDa protein, we used 1,5-difluoro-2,4-dinitrobenzene, with a span of 0.3 nm, the reagent of choice for analysis of nearest-neighbour proteins, in cross-linking experiments. The disappearance of the 41 kDa and 52 kDa protein components is associated with the concomitant appearance of a cross-linked high-molecular-mass complex of about 510 kDa, as is demonstrated by SDS/PAGE (Fig. 5, lanes B–D). An immunoblot with appropriate antibodies proves that this complex consists of both the 41 kDa and 52 kDa proteins (lanes E and F). For a control it was shown that the unmodified enzyme protein could not be cross-linked by 1,5-difluoro-2,4-dinitrobenzene (lane A).

Identity of the mitochondrial 52 kDa protein

The analysis of a partial amino acid sequence of the 52 kDa protein failed, as the *N*-terminal end of the polypeptide chain is apparently blocked. Therefore, after digestion with *S. aureus* V8 proteinase the peptides were separated by SDS/PAGE and blotted on to an Immobilon P membrane for analysis of the amino acid sequence. The information obtained on the sequence of 30 amino acid residues proved them to be a part of the mitochondrial glutamate dehydrogenase with 93% sequence identity [20] (Fig. 6). The modified forms of acetyl-CoA acetyltransferase show also a co-purified catalytic activity (9.3 units/mg of protein) of glutamate dehydrogenase.

DISCUSSION

The co-immunoprecipitation and the cross-linking of the CoA-modified forms of acetyl-CoA acetyltransferase with the glutamate dehydrogenase present evidence for strong physical interactions of these proteins. These interactions exhibit stability in exchange (anion/cation) chromatography and in gel filtration. With respect to enzymic activity (acetyl-CoA acetyltransferase) different molecular masses of 460 kDa and 250 kDa were obtained. Presumably these various association states are caused by interactions of the CoA-modified forms with glutamate dehydrogenase and, possibly, with a 72 kDa protein. When the gel filtration was repeated at the higher pH of 7.4 the putative complexes were resolved and a single activity peak with a molecular mass of 151 kDa was obtained. This molecular mass could correspond to a tetramer of the modified forms with a molecular mass of 151 kDa [21] and a trimer of glutamate dehydrogenase with a calculated molecular mass of 168 kDa [22]. Therefore it might be concluded that the protein complexes obtained at pH 6.8 are mediated through charged groups, which are abolished at the higher pH. However, the specific interactions of the CoA-modified forms with the glutamate dehydrogenase still persist, as following this gel filtration at pH 7.4 these proteins can be mutually immunoprecipitated and can be cross-linked.

The mitochondrial-matrix enzymes acetyl-CoA acetyltransferase and glutamate dehydrogenase are not metabolically linked enzymes. Glutamate dehydrogenase is known to form complexes with several enzymes, e.g. aspartate aminotransferase (EC 2.6.1.1) and carbamoyl phosphate synthase I (EC 2.7.2.5) [23–25]. Apparently the complexes formed provide kinetic advantages [25]. Here we present evidence that a CoA-modified, partially active, protein interacts with glutamate dehydrogenase. It is assumed that the glutamate dehydrogenase also exists in CoA-modified forms. An indication for this is shown by a [^{14}C]pantothenic acid-derived 53 kDa radioactively labelled protein, which has been demonstrated in an autoradiogram of an SDS/PAGE gel of a mitochondrial extract [3]. A putative CoA modification of glutamate dehydrogenase has to be discriminated from the palmitoyl-CoA binding by this protein [24]. We presume, however, that palmitoyl-CoA should be released from the protein during SDS/PAGE and that the observed radioactivity rather refers to tightly bound CoA.

The purified CoA-modified forms of acetyl-CoA acetyltransferase and glutamate dehydrogenase obviously interact with each other and thus appear to be nearest-neighbour proteins. This interaction of metabolically unconnected enzymes might represent the temporary contact of not fully folded, i.e. not fully active, proteins, presumably with another protein(s), in order to achieve the proper folding and assembly to become functionally active. The partial activity of the CoA-modified forms [4], their continually increasing [^{14}C]pantothenic acid-derived radioactivity and their significantly higher [^{14}C]leucine-

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                                   165                              180                               195
GluDH:  R Y S T D V S V D E V K A L A    S L M T Y K [C] A V V [D] V P F G    G A K A G V K I N P K N Y T D
52 kDa:                     V K A L A    S L M T Y K [S] A V V [?] V P F G    G A K A G V K I N P _ _ _ _ _
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Fig. 6. Partial amino acid sequence of the 52 kDa protein aligned with the amino acid sequence of glutamate dehydrogenase (GluDH) predicted from its nucleotide sequence [20]

Residues not shared between the 52 kDa protein and the glutamate dehydrogenase are boxed.

derived radioactivity when compared with the unmodified fully active enzyme [2] support this view.

We are grateful to B. Schmidt, Institut für Biochemie, Georg-August-Universität Göttingen, who performed the amino acid sequencing of the peptide from the 52 kDa protein, to J. G. Sgouros, Martinsrieder Institut für Proteinsequenzen, for alignment of the peptide sequence and to Dr. Hunneman for reading the manuscript. The work contains parts of the Doctoral Thesis of G. S. at the Faculty of Science, University of Göttingen. This investigation was supported by the Deutsche Forschungsgemeinschaft.

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Received 22 February 1991/28 May 1991; accepted 4 June 1991