Immunochemical detection of CoA-modified mitochondrial matrix proteins

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An *in vitro* assay mixture consisting of mitochondrial matrix proteins from rat liver and CoA resulted in the formation of CoA-modified proteins. CoA-modified proteins were demonstrated by detection of CoA. CoA was released from the proteins by dithioerythritol treatment under denaturing conditions and was identified by (a) its retention time on HPLC, (b) its absorption spectrum and (c) its activity in a CoA-specific assay. This CoA represents protein-bound CoA and, in addition, protein-bound palmitoyl-CoA when MgATP was also present in the *in vitro* assay. The detection of immunoreactive proteins using monospecific anti-CoA antibodies exclusively identifies CoA-modified proteins. The specificity of these antibodies can be used to identify both endogenously occurring CoA-modified proteins and proteins that have been modified in the *in vitro* assay. An intact thiol group of CoA is an essential precondition for the modification to occur.

INTRODUCTION

Post-translational modifications of proteins can result in the appearance of distinct forms of an enzyme, and often represent defining steps in protein turnover [1]. The existence of multiple forms has been demonstrated, for example, for 3-oxoacyl-CoA thiolases (EC 2.3.1.16) and for acetyl-CoA acetyltransferase (acetoacetyl-CoA thiolase; EC 2.3.1.9) in mammalian mitochondria [2–4]. The different forms of acetyl-CoA acetyltransferase revealed a charge heterogeneity *in vivo* [5] that could not be traced to different cDNAs [6], but resulted from a CoA modification [7,8]. Thus the fully active unmodified enzyme [9] is sequentially transformed into partially active CoA-modified forms. This modification is accompanied by a drastic decrease in the half-lives of the CoA-modified forms [10].

The administration *in vivo* of [1-^{14}C]pantothenic acid, the precursor of CoA, resulted in the radioactive labelling of several mitochondrial matrix proteins [8]. This radioactivity derived from [1-^{14}C]pantothenic acid could be attributed to protein-bound CoA or acyl-CoA. Acyl-CoA esters have been found to possess distinct binding affinities for the nucleotide binding sites of proteins [11]. In the present paper we describe for the first time the detection of CoA-modified mitochondrial matrix proteins by immunochemical methods. These CoA-modified proteins occurred endogenously and/or were formed in an *in vitro* assay.

MATERIALS AND METHODS

Materials

Materials were obtained from the following sources: CoA, desulpho-CoA, palmitoyl-CoA, dithioerythritol, *N*-ethylmaleimide, haemocyanin, apyrase and SDS/PAGE calibration proteins from Sigma (Deisenhofen, Germany); ATP and adenosine 5′-[β,γ-methylene]triphosphate from Boehringer Mannheim (Mannheim, Germany); Sephadex G25 Superfine and Percoll from Pharmacia-LKB (Freiburg, Germany); HPLC column Supersphere 100 RP-18 from Merck (Darmstadt, Germany); Affi-Gel 10 from Bio-Rad (München, Germany); SEP-PAK C_{18} cartridges and Immobilon-P membranes from Millipore Waters Association (Eschborn, Germany); enhanced chemiluminescence Western blotting reagents, biotinylated molecular mass markers and Hyperfilm from Amersham (Braunschweig, Germany). All other chemicals were of high purity and were obtained from Merck.

Animals

Male Wistar rats were bought from Winkelmann (Borchen, Germany) and were kept on a 12 h day/night rhythm with free access to water and diet from Ssniff (Soest, Germany). The animals were killed under ether anaesthesia and livers were excised immediately.

Isolation of liver mitochondria and preparation of matrix proteins

Mitochondria were isolated at 4 °C using a Sorvall SS34 rotor in a 0.3 M sucrose medium containing 3 mM potassium phosphate buffer (pH 7.2), as described by Huth et al. [7]. The mitochondria were collected by centrifugation at 2100 ***g*** for 10 min. The mitochondrial pellet was washed and resuspended in the above-mentioned isolation medium but containing 30 % (v/v) Percoll and centrifuged for 10 min at 17369 ***g***. The supernatant was removed and the mitochondrial pellet was resuspended in the isolation medium containing in addition 1 mM PMSF, 1 mM EDTA, 1 μg/ml pepstatin and 1 μg/ml leupeptin. Mitoplasts and the mitochondrial matrix fraction were prepared using digitonin (1.2 mg/10 mg of protein) and Lubrol Wx (2 mg/10 mg of protein) as described by Schnaitman and Greenawalt [12] and as modified by Huth et al. [8]. The matrix protein fraction was stabilized by addition of 50 mM potassium phosphate buffer (pH 7.2) and 20 % (w/v) glycerol. Aliquots of this protein fraction were passed through a molecular sieve column of Sephadex G25 Superfine equilibrated with 100 mM Tris/HCl buffer (pH 8.1) before experimental use.

Determination of enzyme activities

Glutamate dehydrogenase (EC 1.4.1.2), NADPH:cytochrome *c* reductase (EC 1.6.99.2) and β-*N*-acetylhexosaminidase (EC 3.2.1.52) were assayed according to Schmidt [13], Sottocasa et al. [14] and von Figura [15] respectively. Acetyl-CoA:orthophosphate acetyltransferase (EC 2.3.1.8) activity was measured as described by Michal and Bergmeyer [16]. Specific activities were defined as μmol of product formed/min per mg of protein.

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Assay of CoA modification of proteins

A sample of 2.0 or 0.5 mg of protein from the mitochondrial matrix protein fraction was incubated in a total volume of 500 μl in the presence of 100 mM Tris/HCl buffer (pH 8.1), 1.5 mM CoA and 5 mM $MgCl_2$, with or without 3 mM ATP. Incubation was at 30 °C for 5 and 30 min respectively, as indicated. The reaction was stopped by transfer on to ice. Each experiment was performed in triplicate.

Preparation of a protein sample free of non-covalently bound compound(s)

For column centrifugation [8,17], a column (5 ml syringe) filled with Sephadex G25 Superfine was equilibrated with 4×2 ml of 20 mM NH_4HCO_3 buffer (pH 8.1) by centrifugation at 450 ***g*** for 3×5 min and then for 10 min. The assay sample (500 μl) was applied to the column by centrifugation at 450 ***g*** for 10 min. The protein fraction was separated successively in two elutions with 200 μl of buffer by centrifugation (450 ***g***, 10 min), with an average protein yield of $81.5 \pm 5.5\%$. This protein fraction was additionally precipitated by trichloroacetic acid at a final concentration of 10 % (w/v) and was pelleted by centrifugation at 8000 ***g*** for 5 min. The proteins were then resuspended by sonication (3×10 s; 160 W) under cooling and washed with 5×1 ml of 5 % (w/v) trichloroacetic acid and 2×1 ml of ether. The protein pellet was solubilized in 20 mM potassium phosphate buffer, pH 5.0 (solvent A), for analysis by HPLC.

The fraction of small compounds was eluted from the Sephadex G25 column by washing with 6×2 ml of 20 mM NH_4HCO_3 buffer (pH 8.1) by centrifugation at 450 ***g*** for 10 min. The eluents were combined, lyophilized and solubilized in solvent A for detection of acyl-CoA by HPLC.

Release of protein-bound small compounds

The protein sample (pellet) prepared by column centrifugation and acid precipitation as described above was solubilized in 750 μl of 20 mM Tris/HCl buffer (pH 8.1) containing 6 M urea, and was incubated for 1 h at 37 °C in the presence of 20 mM dithioerythritol. The proteins were separated by the addition of perchloric acid (0.55 M final concentration), and the perchloric acid was removed as $KClO_4$ by neutralization. Then the supernatant (sample) was placed on a SEP-PAK C_{18} cartridge to remove urea. The compound(s), retained on the cartridge, were eluted with 8 ml of solvent A containing 20 % (v/v) methanol, and were lyophilized and solubilized in 300 μl of solvent A.

HPLC analysis and determination of compounds

The samples were resolved by reverse-phase HPLC on a Merck Supersphere 100 RP-18 125-4 column with an LKB HPLC gradient system. For analytical runs, the column was equilibrated at a flow rate of 1 ml/min in solvent A. Following sample injection, the column was run for 10 min with solvent A and subsequently with a gradient of solvent B as described in the legends to Figures 1 and 2. The total run time, including re-equilibration, was 80 and 50 min for runs with acetonitrile and methanol gradients respectively. CoA and palmitoyl-CoA were quantified using calibration curves of known amounts of pure CoA or palmitoyl-CoA injected directly on to the column. CoA or palmitoyl-CoA when added in amounts as low as 0.1 nmol to the *in vitro* assay was reductively released from the proteins and was obtained as CoA with a recovery of $94.9 \pm 3.1\%$ and $89.2 \pm 3.0\%$ respectively.

Preparation of the protein sample for immunochemical analysis

The protein sample prepared by column centrifugation on Sephadex G25 was incubated for 1 h at 37 °C under denaturing conditions in the presence of 6 M urea but in the absence of dithioerythritol. The protein was pelleted by trichloroacetic acid precipitation, washed and solubilized in SDS sample buffer containing 0.1 % (v/v) Triton X-100.

SDS/PAGE and immunochemical methods

SDS/PAGE

SDS/PAGE under non-reducing conditions was performed according to Laemmli [18] with 12 % (w/v) polyacrylamide. Molecular size markers were phosphorylase *b* (97.4 kDa), BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa) and trypsin inhibitor (20.1 kDa). The proteins were stained with Coomassie Brilliant Blue. For Western blotting, biotinylated molecular mass markers were used.

Preparation of antibodies against CoA

CoA was coupled to haemocyanin as described by Messner et al. [19]. Polyclonal antibodies against haemocyanin-CoA were raised in a rabbit and the IgG fraction was isolated from antisera as described by Huth and Alves [20]. For preparation of anti-CoA IgG, the anti-haemocyanin-CoA IgG was transferred on to a column of Affi-Gel 10 coupled to haemocyanin. The coupling of haemocyanin and the handling of the affinity chromatography column were performed according to the manufacturer's instructions. The anti-CoA IgG passed through the column, and its specificity was evaluated by immunoblotting. Anti-(acetyl-CoA acetyltransferase) antibodies were raised in a rabbit as described by Huth and Alves [20].

Immunoblotting

The discontinuous system was used for semi-dry blotting on Immobilon P membranes [21]. For detection of the CoA-modified proteins, anti-CoA antibodies and the enhanced chemiluminescence amplification system were used.

RESULTS AND DISCUSSION

Among the rat liver mitochondrial matrix proteins showing radioactivity derived from [1-^{14}C]pantothenic acid, the enzymes 3-oxoacyl-CoA thiolase and acetyl-CoA acetyltransferase have been shown to be proteins from which CoA is released by thiol reagents [8]. Clearly, these proteins must undergo modification by CoA, and not by acyl-CoA, as neither of them possesses a nucleotide binding site. For acetyl-CoA acetyltransferase it has been demonstrated that this modification results in a drastic decrease in the half-lives of the modified forms [10]. To detect all CoA-modified proteins among mitochondrial matrix proteins, an *in vitro* CoA modification assay was developed consisting of mitochondrial matrix proteins and CoA, in the presence or absence of MgATP. The protein sample, prepared from rat liver mitochondria, proved to be a pure mitochondrial matrix fraction, as no activity of NADPH:cytochrome *c* reductase (from the endoplasmic reticulum) and only a very low specific activity [$(0.83 \pm 0.27) \times 10^{-3}$ μmol/min per mg of protein] of lysosomal

β-*N*-acetylhexosaminidase could be detected. For comparison, the mitochondrial matrix protein glutamate dehydrogenase exhibited a specific activity of 6.43 ± 1.90 μmol/min per mg of protein.

Identification of the compound released from proteins as CoA

For detection of protein-bound CoA formed in the *in vitro* assay, all small compounds (e.g. free or non-covalently or not tightly bound CoA or acyl-CoA) were separated from the protein sample by column centrifugation using Sephadex G25, and were obtained in the fraction of small compounds. In the presence of CoA and MgATP, 1.96 ± 0.23 nmol of palmitoyl-CoA/5 min per mg of protein could be detected. Obviously, this value represents only a part of the palmitoyl-CoA formed, as palmitoyl-CoA added to the *in vitro* assay was hydrolysed to an extent of $89.7 \pm 10.2\%$ during the preparation of the small compounds. The protein sample prepared by column centrifugation contained no free acyl-CoA molecules, as demonstrated by HPLC (Figure 1A). Commercial pure palmitoyl-CoA exhibited a peak with a retention time of 52 min (Figure 1B). Traces of free CoA (1.2 nmol) still present in the protein sample (results not shown) could be removed completely by an additional trichloroacetic acid precipitation of the protein sample. The HPLC run of this protein sample using a methanol gradient did not exhibit a specific peak of CoA (Figure 1C). CoA has a retention time of 26.4 min under these conditions [8].

Next, the protein sample, free of non-covalently or non-specifically bound CoA or acyl-CoA, was incubated under denaturing conditions with dithioerythritol and the acid-soluble supernatant was analysed by HPLC for released compounds. The run showed a peak with a retention time of 25.8 min (Figure 2A), which is the retention time of pure CoA (Figure 2B). Unequivocal identification of the peak as CoA was provided by its absorption spectrum (Figure 2C) and by its activity in a CoA specific assay using acetyl-CoA:orthophosphate acetyltransferase activity. Under non-denaturing conditions, the thiol reagent failed to release CoA from the proteins (results not shown). Generally, CoA could be released from the protein sample by dithioerythritol only under denaturing conditions.

Specificity and mechanism of the CoA protein modification system

Following incubation of the *in vitro* assay for 5 min in the absence of added CoA, 0.05 ± 0.01 nmol of CoA/mg of protein (Table 1) could be released from the protein sample. Obviously, this CoA is from endogenously occurring CoA-modified proteins. The prior addition of CoA led to a significant increase ($P < 0.001$) in the amount of CoA released from the protein(s), up to 0.57 ± 0.10 nmol/5 min per mg of protein; this indicates the activation of a CoA protein modification system. Under conditions where the mitochondrial matrix protein sample was preincubated (15 min, 30 °C) with apyrase prior to incubation with CoA, the amount of CoA released was not altered. When desulpho-CoA was used instead of CoA, however, the amount of CoA liberated from the proteins was within the range of values obtained for endogenously occurring CoA-modified proteins; this shows that an intact thiol group in CoA is a precondition for this modification. Consequently, the thiol-modifying reagent *N*-ethylmaleimide suppressed the modification by CoA. Thus the *in vitro* modification of proteins by CoA proved to be as thiol-dependent and thiol-labile as the *in vivo* CoA-modified forms of the mitochondrial matrix enzyme acetyl-CoA acetyltransferase [8], suggesting a covalent attachment of CoA to the proteins as a mixed disulphide. The CoA-modified forms of acetyl-CoA acetyltransferase showed an increase in the number of thiol

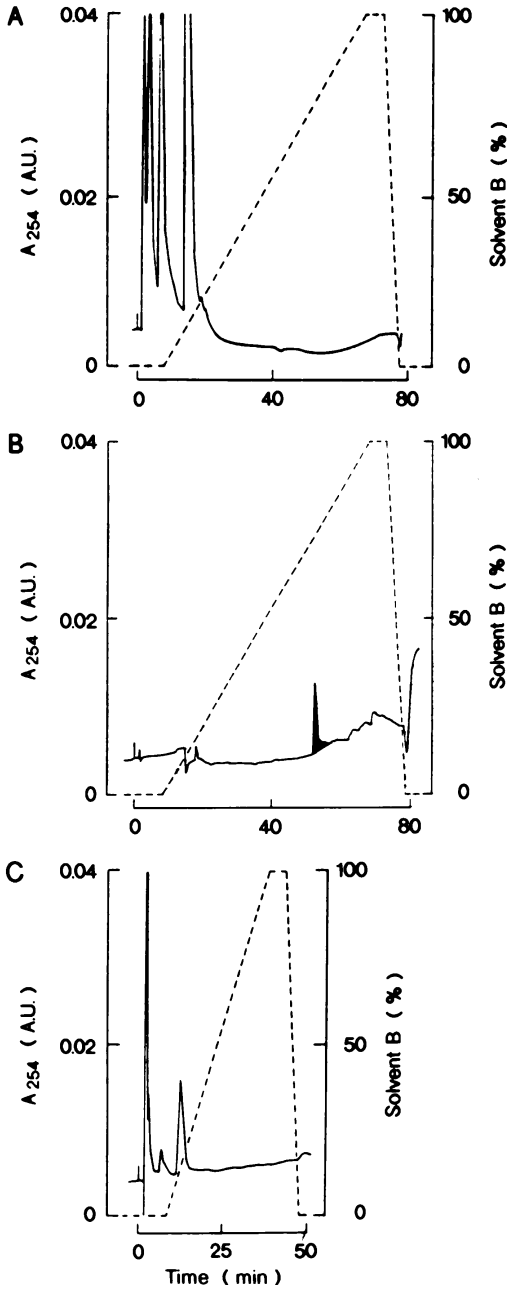

Figure 1 Separation of non-covalently bound acyl-CoA and CoA from mitochondrial matrix proteins as shown by reverse-phase HPLC

Samples of 500 μg of mitochondrial matrix proteins were incubated (5 min, 30 °C) in the presence of CoA and MgATP. To obtain a protein sample containing no free acyl-CoA and CoA, column centrifugation on Sephadex G25 Superfine was performed. For detection of acyl-CoA, the protein sample was solubilized in 20 mM potassium phosphate buffer, pH 5.0 (solvent A), and was applied to a Merck Supersphere 100 RP-18 column, equilibrated with solvent A as described in the Materials and methods section. Runs were performed with a linear gradient of 0–100% solvent B (25% 20 mM potassium phosphate buffer, pH 5.0, 75% acetonitrile). Samples: (**A**) 100 μl (390 μg) of protein; (**B**) 1.0 nmol of pure palmitoyl-CoA for reference. To seperate the free CoA totally, the protein sample prepared by column centrifugation was additionally acid-precipitated, washed and solubilized in solvent A as described in the Materials and methods section. Then the protein sample was applied to the Supersphere 100 RP-18 column. Runs for detection of CoA were performed with a linear gradient of 0–100% of a different solvent B (80% 20 mM potassium phosphate buffer, pH 5.0, 20% methanol). The sample in (**C**) contained 100 μl (402 μg) of protein. Chromatography was monitored at 254 nm (solid lines). A. U., absorbance units.

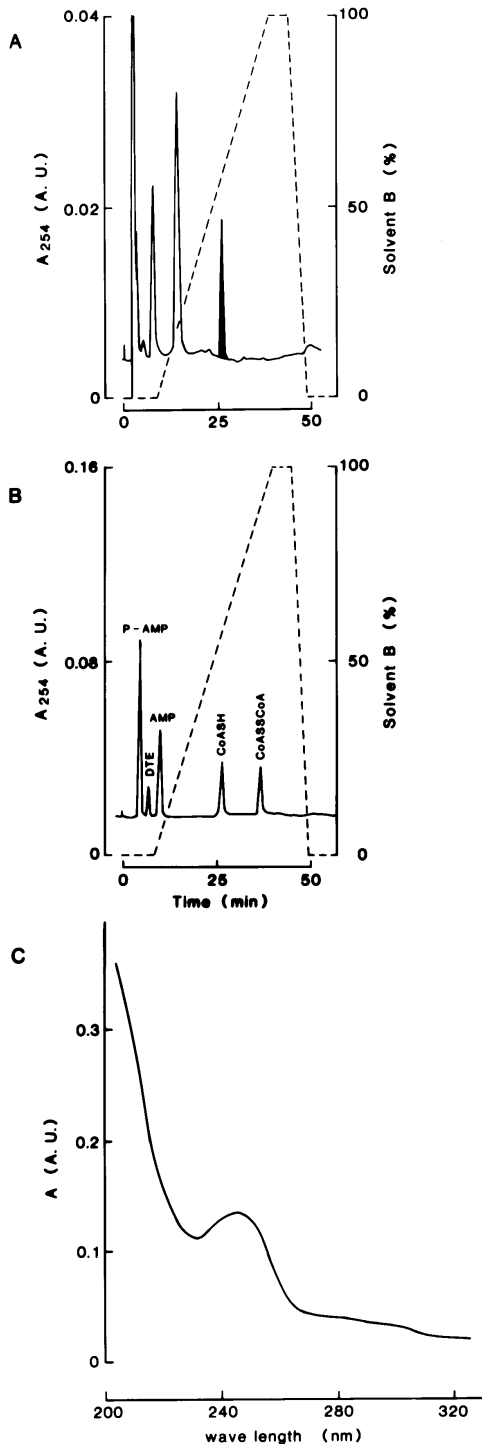

Figure 2 Identification by reverse-phase HPLC and absorption spectrum analysis of the small compound(s) released from proteins

Samples of 2 mg of mitochondrial matrix proteins were incubated (5 min, 30 °C) in the presence of CoA and MgATP. Protein samples were prepared by column centrifugation and acid precipitation, and were solubilized as described in the Materials and methods section. These protein samples, devoid of non-covalently and not-tightly bound CoA, were incubated under denaturing conditions in the presence of 20 mM dithioerythritol. The acid-soluble supernatants were purified on a SEP-PAK C_{18} cartridge and applied to a Merck Supersphere 100 RP-18 column equilibrated in solvent A. HPLC runs were performed with a linear gradient of 0–100% solvent B (80% 20 mM potassium phosphate buffer, pH 5.0, 20% methanol) as indicated. Samples: (**A**) 100 μl of acid-soluble supernatant (total volume 330 μl); (**B**) for reference, 3.8 nmol of adenosine 3′:5′ diphosphate (P-AMP), 872 nmol of dithioerythritol (DTE), 1.9 nmol of AMP, 1.1 nmol of CoA (CoASH) and 0.5 nmol of oxidized CoA (CoASSCoA) were applied to the column. (**C**) Peaks with a retention time of 25.9 min (solid peak, **A**) from several runs, corresponding to about 8 nmol of CoA, were pooled, lyophilized and the absorption spectrum analysed. A, absorbance; A. U., absorbance units.

Table 1 Specificity and mechanism of the CoA protein modification system

Samples of 2 mg of mitochondrial matrix proteins were incubated for 5 min at 30 °C in the presence of additions as indicated: 1.5 mM CoA or desulpho-CoA, 5 units of apyrase, 5 mM *N*-ethylmaleimide (NEM), and 3 mM ATP or adenosine 5′-[β,γ-methylene]triphosphate (p[CH_2]ppA). The protein samples were prepared by column centrifugation and acid precipitation, and protein-bound CoA was reductively released as described in the Materials and methods section. CoA was quantified using an HPLC calibration curve with pure CoA. Values are not corrected for control data in the absence of added CoA, and represent means ± S.D. of the numbers of experiments given in parentheses.

Additions	CoA released (nmol/5 min per mg of protein)
None	0.05 ± 0.01 (5)
CoA	0.57 ± 0.10 (12)
Apyrase + CoA	0.46 ± 0.02 (6)
Desulpho-CoA	0.07 ± 0.02 (9)
CoA + NEM	0.20 ± 0.08 (12)
CoA + MgATP	1.23 ± 0.25 (12)
CoA + p[CH_2]ppA	0,49 ± 0.03 (6)

groups in comparison with the unmodified enzyme [22]. Covalent binding of CoA through a disulphide bond has also been demonstrated for the mutant β-subunit of F_1-ATPase (where a Tyr residue has been replaced by Cys) [23] and for a flavodoxin of *Klebsiella pneumoniae* [24].

MgATP in the presence of added CoA significantly increased ($P < 0.01$) the amount of CoA released from proteins, from 0.57 ± 0.1 to 1.23 ± 0.21 nmol of CoA/5 min per mg of protein (Table 1). This stimulation is dependent on ATP hydrolysis, as became evident when ATP was replaced by the ATP analogue adenosine 5′-[β,γ-methylene]triphosphate ('AMP–PCP'). The effect of MgATP on the release of CoA from protein addresses the question as to whether CoA is additionally bound to proteins in a thioester linkage. On the other hand, the released CoA might originate from acyl-CoA formed during incubation and bound specifically to proteins with nucleotide binding sites [11].

Does the released CoA represent protein-bound CoA?

To examine unequivocally the origin of the released CoA, an immunochemical approach using anti-CoA antibodies was employed. The anti-CoA IgG was prepared from antisera directed against haemocyanin-CoA by affinity chromatography on haemocyanin-coupled Affi-Gel 10. Figure 3 illustrates the specificity of the anti-CoA antibodies. Immunoreactivity could be detected with CoA-coupled BSA (lane 2) and CoA-modified acetyl-CoA acetyltransferase (lane 4), but not with haemocyanin (lane 1) or with BSA (lane 3).

Following incubation of the mitochondrial matrix proteins with CoA, the protein samples were prepared under non-reducing conditions and subjected to immunoblots using anti-CoA antibodies. At least 18 immunoreactive proteins could be detected among the matrix proteins (Figure 4, lane 1), even when the matrix protein sample had been preincubated with apyrase (lane 2). MgATP did not give rise to additional immunoreactive proteins (lane 3). Five endogenously occurring immunoreactive proteins (lanes 4 and 5) with molecular masses of 54, 45, 43, 41

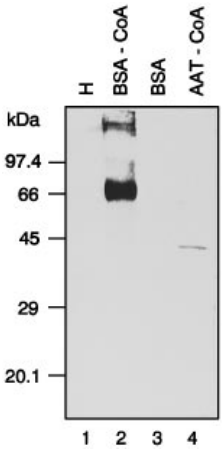

Figure 3 Specificity of anti-CoA IgG

Immunoblot with anti-CoA antibodies of haemocyanin (H), BSA-CoA, BSA and the CoA-modified form (A1) of acetyl-CoA acetyltransferase (AAT-CoA) [8]. Samples of 1 μg of protein were loaded per lane.

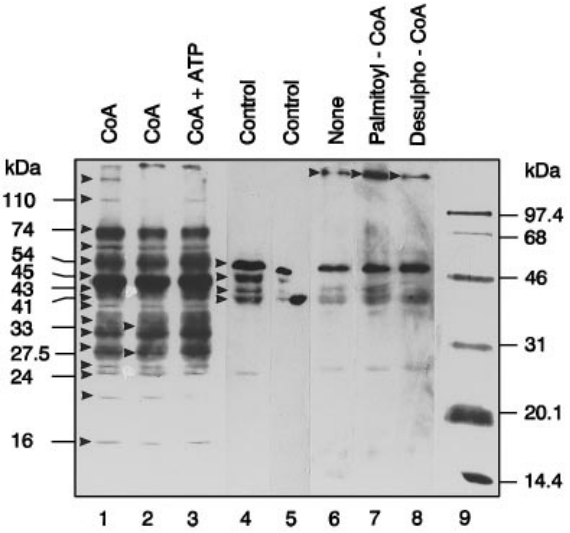

Figure 4 Protein-bound CoA demonstrated by immunoblotting with anti-CoA antibodies

Samples of 2 mg of mitochondrial matrix proteins were incubated (30 min, 30 °C) in the absence or presence of additions as indicated. The protein samples were prepared by column centrifugation on Sephadex G25 and by incubation under denaturing conditions in the absence of dithioerythritol, as described in the Materials and methods section. As a control, mitochondrial matrix proteins were not subjected to incubation and were directly submitted to SDS/PAGE. Samples of 50 μg of mitochondrial protein were transferred to SDS/PAGE under non-reducing conditions and subsequently immunoblotted with anti-CoA antibodies. However, in lane 5 anti-(acetyl-CoA acetyltransferase) antibodies were also applied (see below). Lane 1, 1.5 mM CoA; lane 2, the *in vitro* assay was preincubated (15 min, 30 °C) with apyrase (5 units) prior to incubation with 1.5 mM CoA; lane 3, 1.5 mM CoA + 3 mM MgATP; lane 4, control; lane 5, control, but the lane was cut into two strips: the left was blotted with anti-CoA antibodies, and the right was blotted with anti-(acetyl-CoA acetyltransferase) antibodies; lane 6, no additions; lane 7, 0.1 mM palmitoyl-CoA; lane 8, 1.5 mM desulpho-CoA; lane 9, molecular mass markers.

and 24 kDa were detected directly in the matrix protein sample that was not subjected to incubation. Among these, acetyl-CoA acetyltransferase could be identified as a CoA-modified protein [8] when the sample in lane 5 was submitted to anti-CoA and anti-acetyl-CoA acetyltransferase antibodies in parallel. These endogenously occurring CoA-modified proteins (lane 4) could also be demonstrated, but with slightly lower intensities and an altered pattern (lane 6), when the mitochondrial matrix protein sample was incubated in the absence of CoA and was prepared by the usual procedure. This altered immunoreactive pattern of endogenous CoA-modified proteins, also obtained with palmitoyl-CoA (lane 7) and desulpho-CoA (lane 8), was characterized by a double band of the 41 kDa protein and by the lack of a protein with a molecular mass of 45 kDa. This latter protein appeared as an additional immunoreactive protein with a molecular mass of > 110 kDa (lanes 6, 7 and 8, arrowheads), which is beyond the calibration curve of the molecular mass markers (lane 9). The formation of a higher-molecular-mass species may be due to disulphide bonding of subunits under the non-reducing conditions applied. It is to be stressed that palmitoyl-CoA (lane 7) did not cause the appearance of any extra immunoreactive bands when compared with the endogenously occurring CoA-modified proteins (lane 6). Desulpho-CoA (lane 8), unlike CoA (lane 1), resulted only in the appearance of the endogenous immunoreactive proteins, i.e. an intact thiol group of CoA is an essential precondition for the modification. These results show that the protein-bound CoA detected by immunoreaction with anti-CoA antibodies is covalently bound CoA.

To evaluate the effects of incubation in the absence and presence of CoA on the endogenously occurring CoA-modified mitochondrial matrix proteins, a densitometric analysis of the immunoreactive bands was performed (Figure 4). The amounts of endogenous CoA-modified proteins in the control incubation (lane 4), with molecular masses of 54, 45, 43, 41 and 24 kDa, compared with those in the absence of CoA (lanes 6–8; the 45 kDa protein is replaced by the > 110 kDa protein) were decreased by 17.6 ± 2.3 to 82.4%. On the contrary, incubation with CoA (Figure 4, lane 1) significantly increased the intensity of the immunoreactive 54 kDa protein to $156.3 \pm 1.1\%$ of the control ($P < 0.05$), and that of the proteins of 45, 43 and 41 kDa (which were quantified collectively) to $238.7 \pm 4.3\%$ of control values ($P < 0.001$) (Table 2). The immunoreactivities of the 41 and 24 kDa proteins were apparently not increased. Thus CoA increases the immunoreactivity of three endogenous CoA-modified proteins with molecular masses of 54, 45 and 43 kDa, and causes the appearance of an additional 13 immunoreactive proteins (Figure 4, lane 1).

Table 2 Increases in endogenously occurring CoA-modified mitochondrial matrix proteins on incubation with CoA

Protein bands from three to five immunoblots (like those in Figure 4) were quantified densitometrically using an HP Scan Jet 4c/T scanner and WIN CAM 2.2 gel documentation software (Cybertech, Berlin, Germany). The immunoreactive intensity is expressed as a percentage of that of endogenous immunoreactive proteins present in the mitochondrial matrix protein fraction incubated in absence of CoA; in the latter case, the immunoreactive 45 kDa protein was replaced by the immunoreactive > 110 kDa protein (see Figure 4, lanes 6–8). With respect to the high intensity of the 45 kDa protein (Figure 4, lanes 1–3) the 45, 43 and 41 kDa proteins could not be quantified seperately.

	Immunoreactive intensity (%)	
Proteins (kDa)	CoA	CoA + MgATP
54	156.3 ± 1.1	165.4 ± 4.3
45 + 43 + 41	238.7 ± 4.3	241.4 ± 5.1
24	105.2 ± 2.2	103.1 ± 3.5

Table 3 Detection of CoA-modified mitochondrial matrix proteins using anti-CoA antibodies and by measurement of radioactivity derived from [1-^{14}C]pantothenic acid

Data are taken from Figure 4 (lanes 1, 3, 4 and 6) of the present study, and from Figure 2 (lane F) of Huth et al. [8]. In the latter study, the liver mitochondrial matrix proteins were prepared subsequent to the *in vivo* administration of [1-^{14}C]pantothenic acid to a rat, and the radioactively labelled proteins were revealed by SDS/PAGE and fluorography. The protein of 110 kDa was beyond the limits of the calibration curve of the molecular mass markers.

Molecular mass (kDa)				
In vitro CoA-modified protein detected by:		Endogenously occurring CoA-modified proteins detected by:		
Immunoreactivity		Immunoreactivity		Radioactivity
Lane 1 (CoA)	Lane 3 (CoA + ATP)	Lane 4 (control)	Lane 6 (no additions)	Lane F ([1-^{14}C]pantothenic acid)
> 110	–	–	> 110	–
110	110	–	–	110
–	–	–	–	91
74	74	–	–	–
59	59	–	–	–
54	54	54	54	54
45	45	45	–	45
43	43	43	43	–
41	41	41	41	41
38	38	–	–	–
34	34	–	–	–
33	33	–	–	33
32	32	–	–	–
29	29	–	–	–
27.5	27.5	–	–	27.5
25.5	25.5	–	–	–
24	24	24	24	–
21	21	–	–	–
16	16	–	–	–

MgATP, in the presence of CoA, neither significantly augmented the intensity (Table 2) nor increased the number (Figure 4, lane 3) of immunoreactive proteins. In contrast, MgATP significantly enhanced the amount of CoA released from proteins (Table 1). This suggests that some of the palmitoyl-CoA formed in the *in vitro* assay is bound to proteins with nucleotide binding sites [11] and accounts for the MgATP-dependent increase in released CoA (Table 1). In controls with 50 nmol of palmitoyl-CoA added to the *in vitro* assay, 0.67 ± 0.08 nmol of CoA/mg of protein remained as protein-bound palmitoyl-CoA. Palmitoyl-CoA bound to nucleotide binding sites of proteins clearly remains bound during column centrifugation with Sephadex G25. Thus palmitoyl-CoA did not give rise to a specific peak when the protein sample was analysed by HPLC (Figure 1A). As column centrifugation of the proteins was followed by acid precipitation, the nucleotide binding sites of the proteins were probably destroyed. However, palmitoyl-CoA, when released, was co-precipitated with the proteins by the acid [25], and CoA was reductively released from its binding to proteins and from palmitoyl-CoA. In preparing the protein sample for the immunochemical detection of CoA-modified proteins, the protein-bound palmitoyl-CoA was most likely separated by SDS/PAGE.

Does the *in vitro* CoA modification of proteins have relevance *in vivo*?

Table 3 demonstrates the *in vivo* relevance of the CoA modification of proteins *in vitro*, on the basis of the molecular masses of the *in vitro* and *in vivo* CoA-modified proteins as detected by immunoreactivity with anti-CoA antibodies and by measurement of radioactivity derived from [1-^{14}C]pantothenic acid. It is evident that, of the seven radioactively labelled CoA-modified proteins *in vivo*, three, with molecular masses of 54, 45 or > 110 and 41 kDa, can be detected in the *in vitro* assay as endogenously existing CoA-modified proteins, and six can be detected after an *in vitro* incubation with CoA. As already outlined above, CoA increases the immunoreactivity of endogenous CoA-modified proteins and gives rise to 13 additional immunoreactive proteins. Three of these, with molecular masses of 110, 33 and 27.5 kDa, proved to be also radioactively labelled *in vivo*. The increased number of immunoreactive proteins in the presence of CoA most likely can be traced back to an augmentation of the immunoreactive signal. In addition, it is possible that, as a result of preparation of the protein sample for immunoreactive analysis under non-reducing conditions, some proteins might associate to form higher-molecular-mass species and others may be proteolytically degraded to lower-molecular-mass species, thus giving rise to additional immunoreactive protein bands.

The CoA modification of the mitochondrial matrix enzyme acetyl-CoA acetyltransferase is accompanied by a decrease in its half-life [10]. Methods utilizing the specificity of the anti-CoA antibodies should make the identification of additional CoA-modified mitochondrial proteins feasible and allow the investigation of a possible correlation between CoA modification and the half-lives of these proteins.

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