Turnover and transformation of mitochondrial acetyl-CoA acetyltransferase into CoA-modified forms

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Rat liver mitochondrial acetyl-CoA acetyltransferase (acetoacetyl-CoA thiolase, EC 2.3.1.9) exists additionally in the CoA-modified forms A1 and A2. After a pulse of radioactivity using [³⁵S]methionine in hepatocytes, the highest radioactivity was obtained in the unmodified enzyme. Over the chase time, the radioactivity in the unmodified enzyme decreased, but simultaneously increased in both CoA-modified forms, thus proving that the fully active unmodified enzyme exists before the partially

active modified forms A1 and A2. Also, the specific radioactivity (ratio % radioactivity/% immunoreactive area) of A1 > A2 demonstrates a sequential CoA modification of form A1 to form A2. Acetyl-CoA acetyltransferase was degraded with an apparent half-life of 38.0 h: the modified forms A1 and A2 have half-lives of 24.5 and 7.2 h. The physiological meaning of the CoA modification of acetyl-CoA acetyltransferase is not yet understood.

INTRODUCTION

Most mitochondrial proteins are encoded by nuclear genes, synthesized as precursor proteins in the cytosol and subsequently transported into the organelle. In the matrix, the N-terminal signal sequences of the precursors are proteolytically cleaved and finally the proteins are folded and assembled into functional oligomers [1,2]. The biogenesis of mitochondria, however, includes also the selective degradation of proteins. In addition to degradation of mitochondria by lysosomal autophagy [3], liver cell proteins from different mitochondrial compartments [4] and within one compartment [5] exhibit great differences in their half-lives. The heterogeneity of hepatocytes may share in causing the various turnover rates of proteins [6], but beyond that, these various rates suggest a selective degradation of proteins by mitochondrial proteases [7–9]. Proteases, e.g. an ATP-dependent endoprotease, have been shown to exist in the matrix of mitochondria [7,8].

Little is known about covalent or non-covalent modifications of mitochondrial matrix proteins, which may render them more susceptible to proteolytic attack. For several mitochondrial matrix proteins of rat liver we have demonstrated a transient CoA modification, e.g. of acetyl-CoA acetyltransferase (acetoacetyl-CoA thiolase, EC 2.3.1.9), which catalyses the first step in ketogenesis. Thus the enzyme also exists in the CoA-modified forms A1 and A2 [10,11]. These data prompted us to study the effect of CoA modification on the half-life of this enzyme and to clarify the chronological sequence of the modification. The tool used in this investigation was a pulse–chase experiment using [³⁵S]methionine in a culture of hepatocytes. The unmodified acetyl-CoA acetyltransferase and its CoA-modified forms A1 and A2 were demonstrated by combined cation-exchange chromatography and fused rocket immunoelectrophoresis [10].

MATERIALS AND METHODS

Materials

L-[³⁵S]Methionine was from Amersham Buchler (Braunschweig, Germany). Medium 199 and RPMI medium were from Biochrom K.G. (Berlin, Germany); fetal-calf serum was from Boehringer

(Mannheim, Germany); insulin, penicillin G and streptomycin sulphate were from Serva (Heidelberg, Germany); dexamethasone was from Sigma (Deisenhofen, Germany); Cellex-P cellulose was from Bio-Rad (München, Germany); Protein A–Sepharose CL 4B and agarose A were from Pharmacia–LKB (Freiburg, Germany); Instagel was from Packard Instruments (Frankfurt, Germany); nitrocellulose sheets were from Schleicher & Schüll (Dassel, Germany). All other chemicals were of high purity and were obtained from commercial sources.

Animals

Male Wistar rats were bought from Winkelmann (Borchen, Germany) and kept on a 12 h day–night rhythm with free access to water and a rat standard diet from Ssniff (Soest, Germany).

Pulse–chase experiments

Cell culture

Hepatocytes were isolated by a recirculating perfusion *in situ* with collagenase [12,13]. Cells were suspended in medium M199 supplemented with 4 % fetal-calf serum, 0.1 μ M insulin, 0.1 μ M dexamethasone, 0.17 mM penicillin G and 0.17 mM streptomycin sulphate and incubated at 36.5 °C in an atmosphere containing 5 % (v/v) CO₂ at 90 % humidity of the air. After 3 h the medium was changed and the cells were cultured in RPMI medium free of L-methionine and of fetal-calf serum and incubated for 30 min. For pulse-labelling, L-[³⁵S]methionine (5 μ Ci/dish; sp. radioactivity 70 mCi/mmol) and methionine (final concn. 50 μ M) were added and incubated for a further 1 h. For chase, the cells were washed with 3 $\times$ 2.5 ml medium M199 and then incubated in 2.5 ml of medium M199, supplemented with the above-mentioned additions except calf serum, for the times indicated; the medium M199 was exchanged after 17 h incubation.

Mitochondrial extract

After the times indicated, the harvested cells were washed three times with ice-cold 0.9 % (w/v) NaCl. Cells from a single dish were taken up in 2 ml of ice-cold Hepes buffer (20 mM Hepes,

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220 mM mannitol, 70 mM sucrose, 2 mM Tris, 1 mM EDTA, pH 7.4). Cells from eight dishes were collected and lysed by six strokes of a Dounce homogenizer. Mitochondria were sedimented by centrifugation at 14 600 *g* for 10 min. The pellet was suspended in 1.6 ml of 50 mM potassium phosphate buffer (pH 7.2) containing 1 mM EDTA and 0.5 mM phenylmethanesulphonyl fluoride, and mitochondria were lysed with 0.5% (w/v) Triton X-100 in an incubation (2 h, 4 °C) while stirring. The mitochondrial extract was centrifuged at 100 000 *g* for 1 h.

Cation-exchange chromatography and fused rocket immunoelectrophoresis

The mitochondrial proteins of the cells from 20–30 dishes, containing 171.2 ± 49 μg of acetyl-CoA acetyltransferase as quantified by rocket immunoelectrophoresis [14], were transferred to Cellex-P cellulose columns (1 cm $\times$ 4 cm), which had been equilibrated with 50 mM potassium phosphate buffer (pH 7.2) containing 1 mM EDTA and 20% (w/v) glycerol. The acetyl-CoA acetyltransferase protein was eluted by a K^+ gradient up to 0.65 M K^+ and collected in fractions. These fractions were subsequently analysed for immunoreactive protein by fused rocket immunoelectrophoresis using appropriate concentrations of anti-(acetyl-CoA acetyltransferase) IgG, as described by Huth and co-workers [10,14,15].

Immunoprecipitation

Anti-(acetyl-CoA acetyltransferase) IgG was used in a complex with Protein A–Sepharose CL 4B (20 μg of IgG/ μg of immunoreactive protein). The amounts (μg of immunoreactive protein) of the unmodified enzyme and the modified forms were estimated from the immunoreactive area and the immunospecific amount of acetyl-CoA acetyltransferase protein. Samples of unmodified enzyme and form A1 (20 μg of protein) and of form A2 (6 μg of protein) were immunoprecipitated with suitable amounts of antibody in an incubation at 4 °C for 2 h. The protein–antibody–Sepharose complex was pelleted by centrifugation (10 000 *g*, 5 min) and washed three times with a medium containing 100 mM Tris/HCl (pH 7.2), 100 mM NaCl and 1% Triton X-100 and twice with detergent-free medium. For dissociation the precipitate was suspended in a buffer of 100 mM Tris/HCl (pH 7.2) containing 2% (w/v) SDS and 2% (w/v) mercaptoethanol and heated at 95 °C for 5 min. After centrifugation, 100 μl of supernatant was supplemented with 1.6 ml of scintillation fluid and counted for radioactivity. Control samples omitting the antibody contained an unspecific radioactivity (% of total radioactivity immunoprecipitated in the respective form) of 26.1 ± 12.0 , 35.3 ± 18.5 and 60.0 ± 29.1 % for the unmodified enzyme and for the modified forms A1 and A2 respectively.

SDS/PAGE

SDS/PAGE was performed by the procedure of Laemmli [16]. Calibration proteins were used as described in [17].

Immunoblot

The discontinuous system was used for semi-dry blotting according to the procedure of Kyhse-Anderson [18].

Preparation of antibodies

Antibodies against liver mitochondrial acetyl-CoA acetyltransferase were raised in a rabbit. The IgG fraction was isolated from antisera as described in [19].

RESULTS

To analyse the temporal sequence of the appearance of the unmodified acetyl-CoA acetyltransferase and of its CoA-modified forms, pulse–chase experiments were performed with [^{35}S]methionine in isolated hepatocytes. In addition, the half-life of the acetyl-CoA acetyltransferase was determined.

Demonstration of the acetyl-CoA acetyltransferase protein and immunoprecipitation

The unmodified enzyme and its CoA-modified forms A1 and A2 were isolated from the mitochondrial extract of hepatocytes demonstrated by the two-dimensional method [10] of cation-exchange chromatography and fused rocket immunoelectrophoresis (result not shown). On the basis of the immunoreactive profile, the fractions were pooled for the unmodified enzyme (54.5 ± 5.6 % of immunoreactive area) and for the CoA-modified forms A1 and A2 (35.1 ± 5.1 and 10.4 ± 3.9 % of immunoreactive areas respectively). These amounts of the various

Figure 1 Analysis of immunoprecipitation using anti-(acetyl-CoA acetyltransferase) IgG by SDS/PAGE and immunoblotting

Immunoprecipitations of unmodified enzyme and the CoA-modified forms A1 and A2 using suitable protein solutions corresponding to 20–30 μg of unmodified enzyme, 10–15 μg of form A1 and 5–15 μg of form A2 of immunospecific protein were performed as described in the Materials and methods section. (a) SDS/PAGE. Protein solutions corresponding to samples of (A) 5 μg of unmodified enzyme, (D) 9 μg of form A1 and (G) 10 μg of form A2; immunoprecipitates corresponding to (B) 8 μg of unmodified enzyme, (E) 5 μg of form A1 and (H) 3 μg of form A2; total supernatants after immunoprecipitation of (C) unmodified enzyme, (F) form A1 and (I) form A2; (J) 10 μg of purified acetyl-CoA acetyltransferase. (b) Immunoblot: 5 μg of acetyl-CoA acetyltransferase and protein solutions corresponding to 20 μg of immunoreactive protein were used: (A) acetyl-CoA acetyltransferase (41 kDa); supernatants after incubation with Protein A–Sepharose CL 4B of (B) unmodified enzyme (41 kDa), (D) form A1 (41 kDa) and (F) form A2 (41 kDa); supernatants after immunoprecipitation of (C) unmodified enzyme, (E) form A1 and (G) form A2.

Figure 2 Pulse-chase analysis of the incorporation of [^{35}S]methionine into acetyl-CoA acetyltransferase protein, the unmodified enzyme and the CoA-modified forms A1 and A2

Pulse-chase experiments were performed with hepatocyte cultures (20–30 dishes per experiment). To determine the radioactivity (c.p.m.), immunoprecipitations were done in triplicate. The values of radioactivity (%; **a**) and of specific radioactivity (**b**: ratio % radioactivity/% immunoreactive area) represent means $\pm$ S.E.M. of 3–5 experiments: $\bullet$, unmodified enzyme; $\circ$, modified form A1; $\blacktriangle$, modified form A2.

forms of acetyl-CoA acetyltransferase remained unchanged during the 24 h of primary culture of hepatocytes. On the basis of immunoreactive area a mutual contamination of the various forms could be calculated: the unmodified enzyme was contaminated to the extent of $3.6 \pm 2.6\%$ by form A1, form A1 to $7.2 \pm 4.1\%$ by unmodified enzyme and form A2, and form A2 to $5.1 \pm 4.3\%$ by form A1.

Radioactivity incorporated was determined by immunoprecipitation with anti-(acetyl-CoA acetyltransferase) IgG. In Figure 1(a) it is shown that the unmodified enzyme (lane A) and the CoA-modified forms A1 and A2 (lanes D and G) were specifically (lanes B, E and H) and quantitatively (lanes C, F and I) immunoprecipitated. The traces of immunoreactive protein (41 kDa) in the concentrated total supernatants of forms A1 and A2 immunoprecipitates (Figure 1a, lanes F and I) amounted to 1.0 and 4.3% (densitometric estimation; Figure 1b, lanes E and G) of the total immunoreactive protein. Obviously, this very small amount of immunoreactive protein was released as an antibody-antigen complex from Protein A-Sepharose CL 4B (Figure 1b). In addition, there is no indication of a co-immunoprecipitation of glutamate dehydrogenase (52 kDa protein in SDS/PAGE) with the CoA-modified forms as demonstrated previously [17]. The observed strong physical interactions of glutamate dehydrogenase with the CoA-modified forms were obviously abolished in presence of Triton X-100.

Transformation of acetyl-CoA acetyltransferase into their CoA-modified forms

The transformation of the acetyl-CoA acetyltransferase protein was analysed in pulse-chase experiments. At the given pulse of ^{35}S label (0 h chase) the radioactivity pattern (% of total radioactivity incorporated) revealed the highest incorporation of the label ($75.8 \pm 4.0\%$) into the unmodified enzyme, whereas the modified forms A1 and A2 were labelled only to $21.1 \pm 5.5\%$ and $2.1 \pm 2.7\%$ respectively (Figure 2a). However, over the chase time, the amounts of radioactivity changed reciprocally in the

unmodified enzyme and the modified forms, i.e. the radioactivity decreased to $56.8 \pm 12.1\%$ in the unmodified enzyme and simultaneously increased to $36.2 \pm 10.4\%$ in form A1 and to $13.0 \pm 5.8\%$ in form A2. In the time interval of 24 h, the decrease in label in the unmodified enzyme by 29.0% approximately equals the sum of the increase by 26.4% in form A1 (15.1%) and form A2 (11.3%), thus indicating that 29.0% of the label initially present in the unmodified enzyme was transferred to the modified forms A1 and A2. Obviously, the pulse of radioactivity moves from the unmodified enzyme to the modified forms.

In order to quantify the pulse more closely, we introduced the specific radioactivity, calculated as the ratio % radioactivity/% immunoreactive area (Figure 2b). Thus, if the label was incorporated exclusively into the unmodified enzyme, the ratio should reach the value of 1.84 ($100\%/54.5\%$). After pulsing for 1 h, the unmodified enzyme exhibits a ratio of 1.48 ± 0.11 , which significantly differs from the ratios of 0.60 ± 0.12 ($P < 0.01$) and 0.19 ± 0.23 ($P < 0.01$) for forms A1 and A2 respectively. In addition, the ratio for form A2 is significantly ($P < 0.05$) lower than that of form A1. The data presented in Figure 2 give evidence that the incorporation of [^{35}S]methionine radioactivity into the unmodified enzyme occurs before that of both modified forms, i.e. the unmodified, fully active, acetyl-CoA acetyltransferase precedes the CoA-modified, partially active, forms. In other words, the unmodified enzyme is subsequently transformed into form A1 and then into form A2. If the modified forms would be produced simultaneously, at the pulse, the specific radioactivity (ratio) of form A2 should be higher than that of A1 with respect to its smaller immunoreactive amount. In the chase period, the specific radioactivity increased in both modified forms; however, the lower immunoreactive amount of form A2 gives rise to a steeper increase in the ratio (Figure 2b).

Turnover of the acetyl-CoA acetyltransferase protein

The sequential modification of acetyl-CoA acetyltransferase by CoA into the CoA-modified form A1 and subsequently into

Figure 3 Turnover of acetyl-CoA acetyltransferase and of the CoA-modified forms A1 and A2

Semi-logarithmic plot of % radioactivity against time: ●, unmodified enzyme; ○, modified form A1; ▲, modified form A2. Values of % radioactivity were taken from Figure 2(a). Intersection at 50% radioactivity: (—) unmodified enzyme, 38.0 h; (·····) form A1 and form A2, 38.6 and 36.0 h respectively. Plots represent regression lines with r values of -0.64 , 0.80 and 0.76 for the unmodified enzyme and form A1 and form A2 respectively.

form A2 should proceed with pseudo-first-order kinetics, as the concentration of mitochondrial CoA [B] of 2.4 mM [20,21] drastically exceeds that of acetyl-CoA acetyltransferase [A], of $29.8 \text{ } \mu\text{M}$ ($16.2 \text{ } \mu\text{M}$ unmodified enzyme, $10.5 \text{ } \mu\text{M}$ form A1, $3.1 \text{ } \mu\text{M}$ form A2). The enzyme concentration was calculated on the basis of the immunoreactive amount of $235.1 \pm 29.4 \text{ } \mu\text{g}$ of acetyl-CoA acetyltransferase/g of liver [15] and the rat liver mitochondrial matrix volume of $1.2 \text{ } \mu\text{l/mg}$ of protein [22].

The transformation of acetyl-CoA acetyltransferase proceeds with a velocity of 0.082 nM/s as calculated from the equation $v = k[A]$. The rate constant of $5.07 \times 10^{-6} \text{ s}^{-1}$ obtained from the slope for unmodified enzyme in Figure 3 corresponds to an apparent half-life of 38.0 h . With respect to the unaltered, but different, amounts of the unmodified enzyme and the modified forms A1 and A2, the subsequent transformation steps should continue with an equal velocity but with different rate constants. From these calculated rate constants, half-lives of 24.5 h and 7.2 h were obtained for the modified forms A1 and A2 respectively. The slopes of curves for forms A1 and A2 demonstrated in Figure 3 do not represent their rate constants of transformation ($A1 \rightarrow A2$) or disappearance of A2, but rather they are resultants of the rate constants, e.g. of form A1 formation from the unmodified enzyme and of form A1 transformation to form A2. Thus it becomes plausible that the time required to obtain half of the form A1 radiolabelled (38.6 h , Figure 3) nearly agrees with the half-life (38.0 h) of acetyl-CoA acetyltransferase.

DISCUSSION

The biogenesis of mitochondria includes not only the integration of the imported proteins into their final destination, but also their disintegration, i.e. the selective degradation of different proteins [3–6]. In the matrix of mitochondria, ATP-independent

[23] and ATP-dependent [7,8,24] proteases were demonstrated. Nucleotide specificity of the ATP-dependent protease varies depending on the protein substrates used. Apparently, the protein substrate structure influences the molecular mechanism of proteolysis. Thus the altered conformation of the protein substrate may determine the half-life of a protein, and often a covalent modification of a protein initiates the degradation by cellular proteases [25,26]. Negative ionic charge was among the first properties reported to decrease intracellular stability [27]. The modification *in vivo* by CoA of mitochondrial matrix proteins of rat liver, e.g. of acetyl-CoA acetyltransferase: (a) increases the negative charge of the protein [10]; (b) causes the formation of modified, partially active, forms A1 (72.5% activity) and A2 (53.1% activity) [28]; (c) proved to be acid-stable and thiol-labile [10,11]; and (d) caused a change in the redox state of the modified forms, e.g. the modified form A1 and the unmodified enzyme show thiol group contents of 7.95 ± 0.44 and 9.10 ± 0.51 respectively [29]. The thiol-lability and the increase in the thiol groups of the modified form A1 suggest a covalent attachment of CoA to the protein as a mixed disulphide.

Pulse-chase analysis of the CoA-mediated transformation of acetyl-CoA acetyltransferase indicates that the unmodified enzyme precedes both modified forms A1 and A2 and that these modified forms are formed sequentially. Form A1 is obviously CoA-modified to a lower extent than form A2, as A2 exhibited a 2.3-fold higher [^{14}C]pantothenic acid-mediated radioactivity than A1 [10].

The physiological meaning of the CoA modification of mitochondrial matrix proteins is not yet understood. It has been suggested that the CoA modification, by causing a partial inactivation of acetyl-CoA acetyltransferase, acts in the regulation of ketogenesis. However, the ratio of the fully active unmodified enzyme to the CoA-modified, partially active, forms proved to be unchanged in metabolic situations with increased rates of ketogenesis [14,15]. Acetyl-CoA acetyltransferase was shown to be degraded with a half-life of 38.0 h . Thereby the schedule of transformation into CoA-modified forms excludes the possibility that this post-translational modification participates in the assembly of mitochondrial matrix proteins to functional enzymes [1,2]. This sequential CoA modification rather favours the notion that the CoA-modified forms represent intermediates in an initiating degradation process of the acetyl-CoA acetyltransferase protein: form A1 and form A2 exhibit half-lives of 24.5 and 7.2 h respectively. The modified form A2 may be only or preferentially susceptible to degradation via a mitochondrial protease. Thus the very low immunoreactive amount (10.4% of immunoreactive area) of form A2 in comparison with form A1 and the unmodified enzymes supports this view. However, we are aware of the possibility that a given modification may have little effect on the proteolytic susceptibility of a protein.

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