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Covalent conjugation of mammalian calmodulin with ubiquitin

R. Ziegenhagen, P. Gehrke and H.P. Jennissen

Institut für Physiologie, Physiologische Chemie & Ernährungsphysiologie, Ludwig-Maximilians-Universität München, Veterinärstr. 13, D-8000 München 22, FRG

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In this paper it is shown that mammalian calmodulin from bovine testis is a substrate for reticulocyte ubiquitin conjugating activity (UCA) forming a 1:1 covalent conjugate between bovine calmodulin and ubiquitin (uCaM). There is an absolute requirement for Ca^{2+} in the range of ~ 10 μM for ubiquitination of calmodulin to occur. This novel conjugate (uCaM) shows a Ca^{2+}-dependent mobility change in polyacrylamide gel electrophoresis in the presence of SDS, indicating that the calmodulin-ubiquitin conjugate still retains the mobility change of native calmodulin. This conjugation reaction could be of prime importance for the intracellular turnover of calmodulin in the mammalian cell, although it cannot be excluded that the ubiquitin-calmodulin conjugate might in itself be of biological relevance.

Calmodulin; Ubiquitin; Calmodulin-ubiquitin conjugate; Protein ubiquitination; ATP-dependent proteolysis; Trimethyllysine

1. INTRODUCTION

Ubiquitin is a heat-stable polypeptide (8.5 kDa) which has evolved extremely conservatively [1] (3 amino acid exchanges between yeast and man). It consists of 76 amino acids and is a cofactor of ATP-dependent proteolysis in reticulocytes [2]. In this system ubiquitin is covalently attached ('ubiquitination') via an isopeptide linkage to the protein to be degraded [2]. In contrast to reticulocytes, a ubiquitin-dependent proteolysis system similar to the activity in reticulocytes could not be detected in mammalian striated muscle [3], although a large number of endogenous proteins can be ubiquitinated by this tissue [3].

Several years ago, Gregori et al. [4] reported that mammalian calmodulin which appears to have a high turnover [5] cannot be ubiquitinated by conjugating activity from reticulocytes in contrast to calmodulin from *Dictyostelium discoideum* due to the presence of trimethyllysine residue 115 in mammalian calmodulin which is absent in *Dictyostelium* calmodulin [6]. During routine ubiquitination tests we repeated those experiments [7] with a different outcome.

It will be shown here that mammalian calmodulin from bovine testis can indeed be ubiquitinated by conjugating activity from reticulocytes indicating that the trimethyllysine residue does not protect mammalian calmodulin from being ubiquitinated as was previously believed. It therefore appears pertinent that the role of ubiquitin in the turnover of mammalian calmodulin be reevaluated.

Correspondence address: H.P. Jennissen, Institut für Physiologie, Physiologische Chemie & Ernährungsphysiologie, Universität München, Veterinärstr. 13, D-8000 München 22, FRG

2. METHODS AND MATERIALS

2.1. *Preparative method*

The preparation of reticulocytes, reticulocyte lysate and APF II (ATP-dependent proteolysis fraction II) are described in [3,8]. The specific activity of APF II ($\sim$ 7 mg/ml) was $\sim$ 2.6 arbitrary units/mg. APF II was further purified [7] by affinity chromatography according to [9,10] on ubiquitin-Sepharose ($\sim$ 20 mg/ml packed gel) prepared as in [11]. The eluates from the affinity column containing E1-E3 [9] were pooled, concentrated to $\sim$ 1.5 mg/ml (Centricon tubes, Amicon) and stored in 50 mM Tris-HCl, 0.2 mM DTE, pH 7.2 (affinity purified reticulocyte UCA). Ubiquitin was isolated from pig blood according to [3], passed over a Sephadex G-50 column in 0.1 M acetic acid in the

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last step and taken up in 20 mM Tris, pH 7.5. The biological activity of ubiquitin was tested with APF II as described [3]: The ubiquitin concentration necessary for half-maximal activation of the ATP-dependent protease (maximal activation 7–10-fold) was 1.5–2.0 μM. Bovine testis calmodulin was isolated according to [12] and purified further by affinity chromatography according to [14]. This calmodulin contains 1 mol trimethyllysine per mol [12] as could be reconfirmed (determined by Dr M. Goldberg, this Institute, according to [12]). The biological activity of calmodulin was tested [14] with phosphorylase kinase [15] in the AutoAnalyzer test [16]: The concentration of calmodulin necessary for half maximal activation of phosphorylase kinase (maximal activation 6–7-fold) was 30–50 nM.

2.2. *Analytical methods*

The measurement of ATP/Mg^{2+}-dependent proteolysis activity employing ^{125}I-labelled bovine serum albumin (^{125}I-BSA) as substrate is described in [3]: 1 arbitrary unit corresponds to % proteolysis/h in the incubation mixture. ^{125}I-ubiquitin ($2–8 \times 10^5$ cpm/μg) was labelled with ^{125}I according to the chloramine-T procedure [3,17]. The conjugation of proteins with ^{125}I-ubiquitin and subsequent autoradiography were performed as in [3]. As protein substrates bovine pancreatic RNase A (Sigma) oxidized according to [10] at 0.4 mg/ml (32 μM), lysozyme from egg white (Fluka) [18], at 0.4 mg/ml (30 μM), cytochrome *c* from *Candida krusei* (Sigma) (see [9]), at 0.4 mg/ml (32 μM) and calmodulin, at 0.9 mg/ml (53 μM; see also [4]) (final concentrations) were incubated with the ubiquitin-conjugating activity (UCA [7]) of APF II (final concentration 0.88 mg/ml) and affinity-purified UCA (final concentration 0.22 mg/ml) as described [3]. Except for calmodulin these substrates have a free N-terminal amino group [19]. In the case of calmodulin, 10 μM $CaCl_2$ was added to the incubation mixture; controls were run with 1 mM EGTA. Ubiquitin-conjugating activity was quantitated by laser densitometry Ultroscan (Pharmacia-LKB, Freiburg) of autoradiograms obtained by electrophoresis (see below). One unit of enzyme activity is defined according to Hershko et al. [10] as the amount of enzyme required for the incorporation of 1 μmol ^{125}I-ubiquitin into conjugates/min under the conditions employed. Protein [20] was determined on an AutoAnalyzer I (Technicon).

After 60 min at 37°C [3] incubation mixtures (100 μl, see above) were stopped by the sequential addition of 20 μl mercaptoethanol, 20 μl SDS (10%) and 25 μl sample buffer [21], heated to ~ 100°C for 3–5 min until the volume was reduced to ~ 70 μl and then applied to polyacrylamide gels (4–20% gradient or 15% gels) in the presence of SDS [21]. For analysis of Ca^{2+}-dependent mobility changes of calmodulin and derivatives [22] the incubation mixtures were stopped and concentrated by trichloroacetic acid precipitation by adding 20 μl mercaptoethanol and 1/10th volume of 50% trichloroacetic acid. The trichloroacetic acid pellet was neutralized with a few microliters of 2 M Tris and taken up in ~ 70 μl sample buffer [21] containing either (final concentration) 800 μM $CaCl_2$ ($+Ca^{2+}$) or 10 mM EGTA ($-Ca^{2+}$). Molecular mass standards for polyacrylamide gel electrophoresis in the presence of SDS were phosphorylase *b* (97 kDa) [23], BSA (66 kDa) [24], creatine kinase (40 kDa) [25], chymotrypsinogen (25 kDa) and calmodulin (17 kDa) [26]. Qualitative autoradiographic analysis of electrophoresis gels in the presence of enhancer was performed (film exposure 4 h at −80°C, processing at room temperature) as described in [3]. For the quantitative assay of conjugating activity (see above) calibration curves were run by applying specific amounts (3800–63 000 cpm) of ^{125}I-ubiquitin (2.67×10^5 cpm/μg) to polyacrylamide gradient gels (4–20%) [21] followed by autoradiography (exposure of film to the gel at −80°C (see above) for 15 h) and development at 4°C. The darkening obtained on the film (maximum 1.8 *A*) was scanned on a laser densitometer and the peak area was graphically determined (see Fig.3 below).

3. RESULTS AND DISCUSSION

3.1. *Ubiquitination of control proteins*

Ubiquitin-conjugating activity (UCA) of APF II (lane 6) and of affinity-purified UCA lanes (1–5) is shown in fig.1 employing the substrates oxidized RNase (19.7 kDa), lysozyme (14.3 kDa) and cytochrome *c* (12.5 kDa). Unconjugated oxidized RNase always ran with a higher electrophoretic molecular mass (18–20 kDa) than was expected from the sequence molecular mass (12.6 kDa). The first two major conjugates of oxidized RNase (fig.1, lane 1) show molecular masses of 27.1 and 32.1 kDa. The first two conjugates of lysozyme (fig.1, lane 3) correspond to 22.3 and 27.5 kDa. Cytochrome *c* (fig.1, lane 4) shows conjugate molecular masses of ~ 21 and 29.3 kDa. The addition of 1 mM EGTA to the incubation mixture has

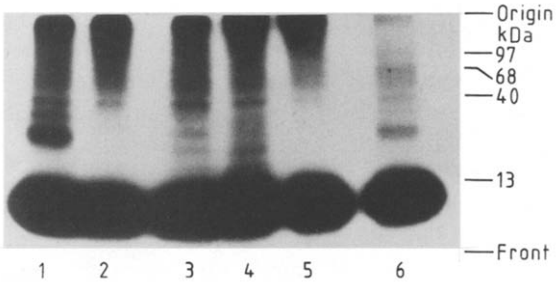

Fig.1. ATP-dependent formation of covalent conjugates between ^{125}I-ubiquitin and various purified proteins by ubiquitin conjugating activity from reticulocytes. An autoradiogram of the electrophoretic separation of ^{125}I-ubiquitin conjugates of the substrates oxidized RNase, lysozyme and cytochrome *c* on a polyacrylamide gradient (10–20%) gel in the presence of SDS according to Laemmli [21] is shown. Unconjugated ^{125}I-ubiquitin (2.7×10^5 cpm/μg) runs at the buffer front. For further details and the molecular mass standards employed (not shown) see text. The applied samples contained: lanes: (1) 40 μg oxidized RNase + 2.5 μg ^{125}I-ubiquitin + 25 μg affinity-purified UCA; (2) control: 2.5 μg ^{125}I-ubiquitin + 25 μg affinity-purified UCA; (3) 40 μg lysozyme + 2.5 μg ^{125}I-ubiquitin + 25 μg affinity-purified UCA; (4) 40 μg cytochrome *c* + 2.5 μg ^{125}I-ubiquitin + 25 μg affinity-purified UCA; (5) control: 2.5 μg ^{125}I-ubiquitin + 25 μg affinity-purified UCA; (6) reference: 40 μg oxidized RNase + 2.5 μg ^{125}I-ubiquitin + 88 μg APFII.

no influence on the ubiquitination of these substrates (not shown). The large band at the bottom of the autoradiograms corresponds to the free ^{125}I-ubiquitin and can vary according to the specific activity and film exposure.

For the substrate RNase (27 kDa conjugate) ubiquitin-conjugating activity was measured by quantitative autoradiography (see section 2 and fig.3 below) amounting to a specific conjugating activity of ~ 2.7 μU/mg for APF II and 19.0 μU/mg for affinity-purified UCA. The total amount of RNase converted in 60 min lies between 0.4 and 0.8%. A comparison of the above specific activities with the data of Hershko [9,10] is difficult, since under our conditions the specific activity of APF II is already ~ 100-fold higher than the corresponding activity described in [10]. The difficulties involved here can be surmised by the fact that even for purified component E3 the specific activities reported vary between 0.65 [10] and 200 or more [9] μU/mg.

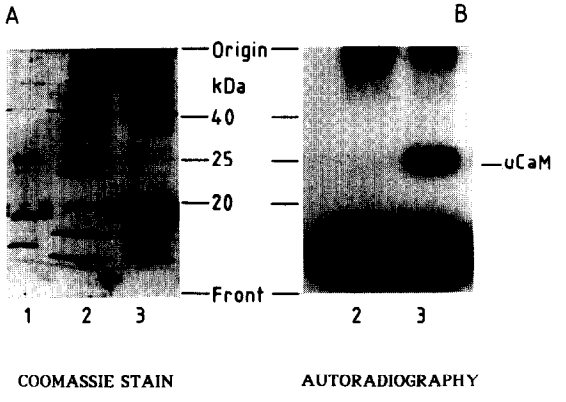

Fig.2. ATP-dependent conjugation of ubiquitin to bovine testis calmodulin by ubiquitin-conjugating activity from reticulocytes. An autoradiogram of the electrophoretic separation of the ^{125}I-ubiquitin-calmodulin conjugate from the free substrates on a 15% polyacrylamide gel in the presence of SDS according to Laemmli [21] is shown. ^{125}I-ubiquitin (spec. act. 7.69×10^5 cpm/μg) was conjugated to calmodulin by affinity-purified UCA in the presence of ~ 10 μM $CaCl_2$. Molecular masses were calibrated with chymotrypsin (25 kDa, lane 1), creatine kinase (40 kDa, lanes 2,3), calmodulin (17 kDa, lane 3) and parallel runs (not shown). For further details on the incubation mixture and autoradiography see legend to fig.1 and text. (A) Coomassie blue-stained gel, (B) autoradiogram. The applied samples contained: lanes: (1) 10 μg chymotrypsin (25 kDa, with minor bands); (2) control: 2.3 μg ^{125}I-ubiquitin + 25 μg affinity-purified UCA; (3) 90 μg calmodulin + 2.3 μg ^{125}I-ubiquitin + 25 μg affinity-purified UCA.

3.2. *Ubiquitination of calmodulin*

Incubation of affinity-purified UCA with purified bovine testis calmodulin and ^{125}I-ubiquitin (fig.2) leads to the formation of a novel radioactive band with a molecular mass of ~ 26–28 kDa (fig.2B, lane 3) in agreement with the conclusion that a covalent 1:1 ubiquitin-calmodulin conjugate (uCaM) has been formed. For the trimethyllysine-free *Dictyostelium* ubiquitin-calmodulin conjugate a value of ~ 26 kDa was found [4]. In the absence of either bovine calmodulin or ATP no conjugate is formed (fig.2B, lane 2). In the presence of 1 mM EGTA ($-Ca^{2+}$) in the incubation mixture also no conjugate is formed (same picture as lane 2 in fig.2B, not shown). High molecular mass conjugates of endogenous substrates remaining at the top of the separation gel are not specific for calmodulin (see control lane 2, fig.2B).

The ubiquitin-conjugating activity of APF II and affinity-purified UCA was also determined for the substrate calmodulin in the presence of Ca^{2+} yielding specific conjugating activities of 2.2 μU/mg with APF II and 3.4 μU/mg respectively (see also fig.3 below). In the presence of 1 mM EGTA only background activity can be detected.

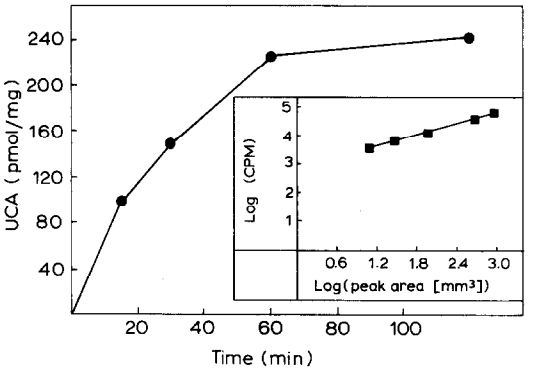

Fig.3. Progress curve of the conjugation of calmodulin with ^{125}I-ubiquitin as derived from quantitative autoradiography. The incubation mixture for the conjugation of ^{125}I-ubiquitin with calmodulin by affinity purified UCA (1.3 mg/ml) in the presence of ~ 10 μM $CaCl_2$ is described in the text. Electrophoresis was performed in the presence of SDS on a polyacrylamide gradient (5–20%) gel [2]. (Inset) Calibration curve for quantitation of autoradiograms: specific amounts (~ 3800–63 000 cpm) of ^{125}I-ubiquitin (2.67×10^5 cpm/μg) were elctrophoresed on polyacrylamide gradient gels (4–20%) according to Laemmli [21]. The darkening obtained on the film (maximum 1.8 *A*) was scanned on an LKB laser (659 nm) densitometer (Ultroscan, Pharmacia-LKB, Freiburg), full scale 2.0 *A*, and the peak area was graphically determined. For further details see text.

The total amount of calmodulin converted in 60 min lies between 0.1 and 0.2%. In the case of APF II these results demonstrate that under our conditions mammalian calmodulin is equally well conjugated as RNase.

Fig.3 illustrates that the conjugation of calmodulin with ubiquitin (affinity-purified UCA) is a time-dependent reaction showing a saturation phenomenon (0.1% calmodulin converted) after ~ 60 min. The specific activity for UCA at 60 min with calmodulin is 3.8 μU/mg. If the initial rates are taken (first 15 min) specific activities over 6.6 μU/mg are obtained.

Fig.4 demonstrates that the ubiquitin-calmodulin conjugate also displays a Ca^{2+}-dependent electrophoretic mobility change in the presence of SDS thereby identifying the conjugate as having calmodulin as one component and ubiquitin (^{125}I) as the other (fig.4B, lanes 2–4). In the presence of EGTA unmodified calmodulin runs as a single band (lane 5) with an apparent molecular mass of 19.8 kDa in agreement with the data of Clark et al. [27]. In the presence of Ca^{2+} two bands can be seen (lanes 6,7) running with apparent molecular masses of 18.0 and 16.6 kDa, respectively, which apparently correspond to different Ca^{2+}-forms of calmodulin. Clark et al. [27] reported values of ~ 15 kDa for the Ca^{2+}-form. Analogously, in the presence of EGTA (fig.4B, lane 4) the major ubiquitin-calmodulin conjugate band runs at 29.3 kDa. In this case a minor band of ~ 27 kDa can also be detected. In the presence of Ca^{2+} (fig.4B, lanes 2,3) two ubiquitin-calmodulin conjugate bands at ~ 28 (minor band) and 26.7 kDa (major band) are found. The magnitude of the mobility change of the major bands of the ubiquitin-calmodulin conjugate (fig.4B, lanes 2–4) corresponds to a maximum change in molecular mass of ~ 3 kDa which agrees well with the maximum molecular mass change of free calmodulin (fig.4A, lanes 5–7).

Gregori et al. [4] concluded that trimethyllysine protects mammalian calmodulin from being ubiquitinated and from degradation of ATP-

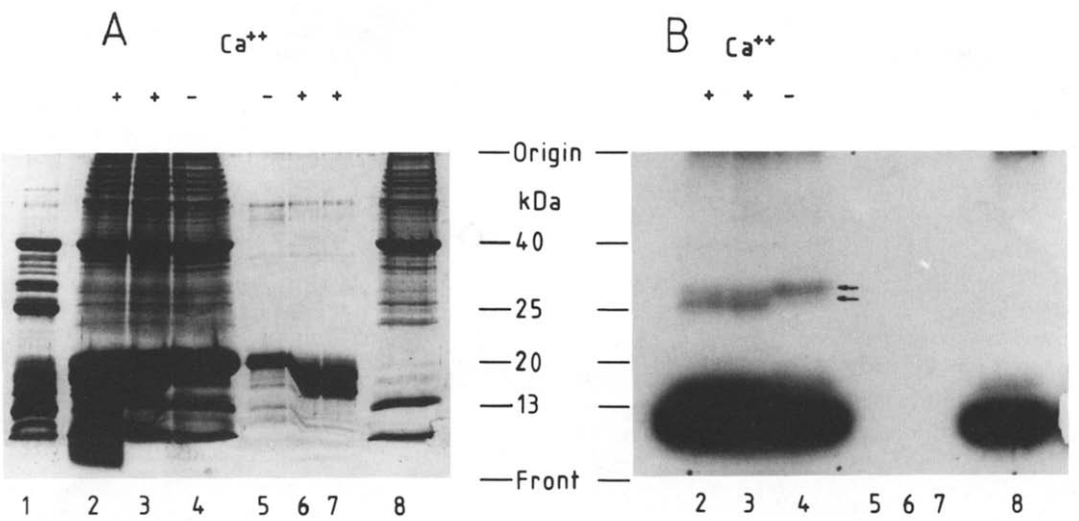

Fig.4. Ca^{2+}-dependent mobility change of ubiquitin-calmodulin conjugate (uCaM) as monitored by polyacrylamide electrophoresis in the presence of SDS. An autoradiogram of the electrophoretic separation of a ^{125}I-ubiquitin-calmodulin conjugates from the single substrate proteins on a 15% polyacrylamide gel in the presence of SDS according to Laemmli [21] is shown. ^{125}I-ubiquitin (spec. act. 1.88×10^5 cpm/μg) was conjugated to calmodulin by affinity-purified UCA in the presence of ~ 10 μM $CaCl_2$. The molecular masses were calibrated with the standards in lane 1, calmodulin and parallel runs. For further details on the incubation mixture and autoradiography see text. (A) Coomassie blue-stained gel, (B) autoradiogram: the arrows indicate the ^{125}I-ubiquitin-calmodulin conjugate. The applied samples contained: lanes: (1) calibration proteins (creatine kinase 40 kDa, chymotrypsinogen 25 kDa, cytochrome *c* 12.5 kDa); (2) 90 μg calmodulin, 2.5 μg ^{125}I-ubiquitin, 25 μg UCA, 0.8 mM Ca^{2+}; (3) 90 μg calmodulin, 2.5 μg ^{125}I-ubiquitin, 25 μg UCA, 0.8 mM Ca^{2+}; (4) 90 μg calmodulin, 2.5 μg ^{125}I-ubiquitin, 25 μg UCA, 10 mM EGTA; (5) control: 10 μg calmodulin, 10 mM EGTA; (6) control: 10 μg calmodulin, 0.8 mM Ca^{2+}; (7) control: 10 μg calmodulin, 0.8 mM Ca^{2+}; (8) control: 2.5 μg ^{125}I-ubiquitin, 25 μg affinity-purified UCA.

dependent protease [4]. In this paper we show that ubiquitin-conjugating activity from reticulocytes (APF II) will in fact covalently couple ubiquitin to purified bovine testis calmodulin at rates comparable to the ubiquitination of oxidized RNase. Since it was concluded that the bovine brain and testis calmodulins have an identical sequence [12] the difference between our finding and that of Gregori et al. [4] cannot be due to the organ employed as calmodulin source. Since the same source (i.e. reticulocytes) was employed for the preparation of ubiquitin-conjugating activity it is very improbable that calmodulin is demethylated to any extent by reticulocyte enzymes present in APF II. If this had been the case Gegori et al. [4] should also have detected ubiquitination of bovine calmodulin. In this context it is of interest that calmodulin, which possesses a blocked (acetylated) N-terminal amino acid (alanine) [26], is a good substrate for UCA. It has been reported for other proteins that this modification is an inhibitory signal to ubiquitination [19]. A very interesting fact is that the Ca^{2+}-dependent mobility change of calmodulin is retained after conjugation, identifying it as a Ca^{2+}-binding conjugate. Since other ubiquitinated proteins also appear to occur independently from proteolytic breakdown (e.g. ubiquitin-H2A semihistone [28] or lymphocyte homing receptor [29]) the ubiquitin-calmodulin conjugate might also have a biological function. Another indication for the biological relevance of the ubiquitination of calmodulin is the absolute dependence on Ca^{2+}. If, on the other hand, ubiquitination of calmodulin is the initial step in calmodulin breakdown (which might explain the minor band in the presence of EGTA, fig.4B, lane 4), then this reaction would suggest a mechanism for the selective breakdown of this important protein which appears to have a very high turnover in skeletal muscle [5].

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