Volume 315, number 3, 347–352 FEBS 12009 January 1993

A ubiquityl-calmodulin synthetase that effectively recognizes the Ca^{2+}-free form of calmodulin

M. Majetschak, M. Laub and H.P. Jennissen

Institut für Physiologische Chemie, Universität-GHS-Essen, Hufelandstr. 55, D-4300 Essen 1, Germany

Received 26 October 1992; revised version received 3 December 1992

Ubiquityl-calmodulin synthetase (uCaM-synthetase) activity as detected in reticulocyte lysate and the crude extracts of rabbit tissues [FEBS Lett. 294 (1991) 229–233] has been well characterized as being essentially Ca^{2+}-dependent ($-Ca^{2+}/+Ca^{2+}$ activity ratio: 0.15–0.2). However, during the purification of this enzyme on ubiquitin-Sepharose the Ca^{2+}-dependent activity is lost and an essentially Ca^{2+}-independent enzyme ($-Ca^{2+}/+Ca^{2+}$ activity ratio: 1.0–1.5) is obtained which was purified 90-fold (uCaM-Syn F1) to a final specific activity of 0.32 pkat/mg. During the purification procedure a second protein factor (uCaM-Syn F2) was isolated that has no catalytic activity by itself but restores Ca^{2+} dependence to the uCaM-Syn F1 fraction ($-Ca^{2+}/+Ca^{2+}$ activity ratio: 0.1) and enhances the catalytic activity in uCaM-Syn F1 in the presence of Ca^{2+} over 40-fold. It is concluded that several (possibly interdependent) forms of uCaM-synthetase exist which display different substrate specificities for calmodulin.

Calmodulin; Ubiquitin; Ubiquityl-calmodulin synthetase; Protein ubiquitination; Calcium

1. INTRODUCTION

In 1987 we reported that a large number of proteins in cardiac and skeletal muscle of the frog, mouse, rat and rabbit can be covalently coupled to ubiquitin in crude extracts [1]. In the same year we were able to identify one of these proteins in the reticulocyte system as calmodulin [2]. In the past years we have reported details on the covalent modification of vertebrate [2–7], plant, fungus [8] and yeast [9] calmodulins with ubiquitin by the enzyme ubiquityl-calmodulin synthetase (uCaM-synthetase). One of the major biologically relevant characteristics of this reaction is the dependence on μM Ca^{2+} concentrations [3,4]. In fact, until now calmodulin is the only protein where ubiquitination is regulated by a second messenger. Employing a specific affinity-based assay [4,9] Ca^{2+}-dependent uCaM-synthetase activity has been detected in most tissues of the rabbit [7,10] and also in the simple eukaryote, yeast (*Saccharomyces cervisiae*) [9] indicating that on the evolutionary time scale this conjugation reaction is at least one billion years old [11,12]. The enzyme is specific for the conjugation of ubiquitin to a single lysine residue [6,8,9] in free unbound calmodulin [4] and initiates a sequence of reactions in which up to three or more ubiquitin molecules are coupled probably yielding a polyubiquitin tail linked to calmodulin [6]. Biologically, ubiquitination might target calmodulin towards proteolysis [5] or to a distinct reaction site in the cell where a reversible modification reaction of calmodulin is decisive [8].

In gel filtration experiments of a DEAE enriched reticulocyte lysate fraction (APF II) it was noted that a minor Ca^{2+}-independent form of uCaM-synthetase activity, which was described previously [8,13], can be separated from the Ca^{2+}-dependent activity. In this paper it will be shown that these two forms of uCaM-synthetase can be fully separated from each other on ubiquitin-Sepharose. The protein conveying Ca^{2+}-dependent activity is not adsorbed to ubiquitin-Sepharose whereas a Ca^{2+}-independent form of the enzyme can be adsorbed and desorbed from the affinity column.

Correspondence address: H.P. Jennissen, Institut für Physiologische Chemie, Universität-GHS-Essen, Hufelandstr. 55, D-4300 Essen 1, Germany.

2. MATERIALS AND METHODS

2.1. *Preparative methods*

The preparation of reticulocytes and the corresponding lysate are described in [1]. Reticulocyte APF II (ATP-dependent proteolysis fraction II) was prepared at 5°C as described in ref. [1,14], except that the lysate (ca. 300–400 ml) was applied to a Fractogel-EMD-DEAE 650 S Superformance column (1.5 cm i.d. × 1.0 cm gel height; Merck Darmstadt) instead of to DEAE Sephacel (Pharmacia). The column was eluted in one step by addition of 350 mM KCl to the buffer as described [1] and pooled (ca. 40 ml). The pool was dialyzed against 20 mM Tris-HCl, 1 mM DTE, 5 μg/ml leupeptin, pH 7.6 (Buffer A) for 4 h at 5°C and stored frozen (APF II, 8 mg/ml).

Reticulocyte APF II was further purified by affinity chromatography according to [2,15] on ubiquitin-Sepharose (17–20 mg/ml packed gel, column: 0.8 cm i.d. × 10 cm). The column was equilibrated with 10 column volumes 50 mM Tris-HCl, 0.2 mM DTE, 0.1 mM EDTA, pH 7.2 (Buffer B). 80 mg of APF II in 10 ml was applied to the column and the run-through (APF IIa) was collected. After washing the ubiquitin-Sepharose (ub-Sepharose) with several volumes 50 mM Tris-HCl, 2 mM DTE, pH 9.0 (Buffer C) and 5–10 volumes of 50 mM Tris-HCl, 1 M KCl pH 7.2 (Buffer D) for regeneration the column was

equilibrated with 50 mM Tris-HCl, 0.2 mM DTE, 2 mM ATP, 5 mM $MgCl_2$, 5 μg/ml leupeptin, pH 7.2 (Buffer E). The run-through (APF IIa) was concentrated to 10 ml and dialyzed on Centricon 30 ultrafilters (Amicon) into Buffer E for the affinity adsorption step. After application of APF IIa to the regenerated ubiquitin-Sepharose column (see above) and washing out the unadsorbed protein (which was reconcentrated to the original volume, uCaM-Syn F2) with Buffer E the column was eluted with 50 mM Tris-HCl, 0,1 mM EDTA, 10 mM DTE, pH 9.0 (Buffer F). The eluate (uCaM-Syn F1) was concentrated and dialyzed against 50 mM Tris-HCl, 0.5 mM DTE, 5 μg/ml leupeptin, pH 7.2 (Buffer G). APF II, uCaM-Syn F1 and uCaM-Syn F2 were stored frozen at −80°C. All steps were performed at 5°C.

Gelfiltration of APF II was performed on a column (1 cm i.d. × 48 cm bed height) of Superose 6 pg (Pharmacia) at 5°C in 20 mM Tris-HCl, 150 mM NaCl, 150 mM Glucose, 1 mM DTE, pH 7.6 (Buffer H).

Ubiquitin was purchased from Sigma (Munich). [^{125}I]CT-ubiquitin was synthesized (50–200 cpm/pmol) by the chloramine-T procedure [1,16]. Bovine testis calmodulin was isolated according to [17] and purified further by affinity chromatography according to [18] and tested as described in [9].

2.2. *Analytical methods*

Reticulocyte uCaM-synthetase was quantitated with the FP-test [4,9]. The incubation mixture (of 50–100 μl) contained 50 mM Tris-HCl, 1 mM DTE, 5 mM $MgCl_2$, 1 mM ATP, 10 mM phosphocreatine, 0.1 mg/ml creatine kinase, 500 μg/ml calmodulin, 1 mg/ml [^{125}I]CT-ubiquitin (specific radioactivity 50–200 cpm/pmol), 0.9 mg/ml reticulocyte APF II or a protein fraction as indicated. The mixtures with calcium (+Ca^{2+}) contained 1.1 mM $CaCl_2$ and 1 mM EGTA. The mixtures without calcium (−Ca^{2+}) only contained 1 mM EGTA. Controls simulating the absence of ATP (i.e. addition of 10 mM EDTA [1]) were run when −Ca^{2+}/+Ca^{2+} activity ratios were determined. After incubation at 37° for 60 min (other times are indicated in the legend) the mixture was heated to 100°C for 5 min. The samples were placed on ice for 5 min, the denatured protein was spun down on an Eppendorf centrifuge and the supernatant adsorbed to Fluphenazine-Sepharose (FP-test) and eluted for analysis as described previously [4,9]. Unless otherwise stated enzymatic activity is expressed in katal. The incubation mixtures for the autoradiographic analysis of uCaM on polyacrylamide gels were the same as those for the FP-test. Electrophoresis of the supernatants (see above) in the presence of SDS was performed on 15% polyacrylamide gels according to [19]. Unless otherwise stated the sample buffer [19] contained 10 mM EGTA [4]. For autoradiography the undried gels were placed between two layers of plastic foil in the presence of enhancer foils (Cronex Lightning Plus, Dupont) and were exposed to X-ray films (Fuji RX, NIF) at −80° C for 24–96 h as required and developed as described [1,3,4]. Protein [20] was determined on an AutoAnalyzer II (Technicon) employing bovine serum albumin as standard.

3. RESULTS

First indications for several forms of uCaM-synthetase came from gel filtration experiments. A typical run on Superose 6 pg is shown in Fig. 1 where APF II was applied to the column. As indicated in the panel of Fig. 1 the −Ca^{2+}/+Ca^{2+} activity ratio increases from 0.02

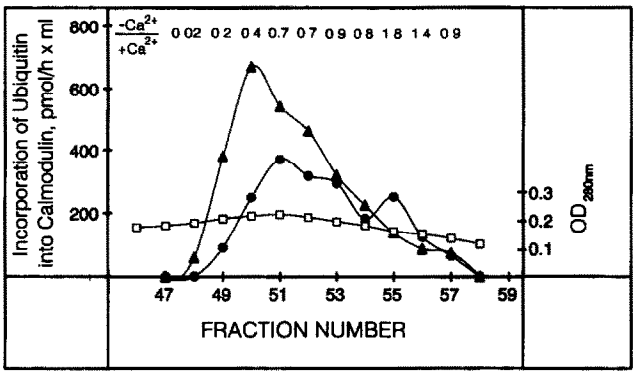


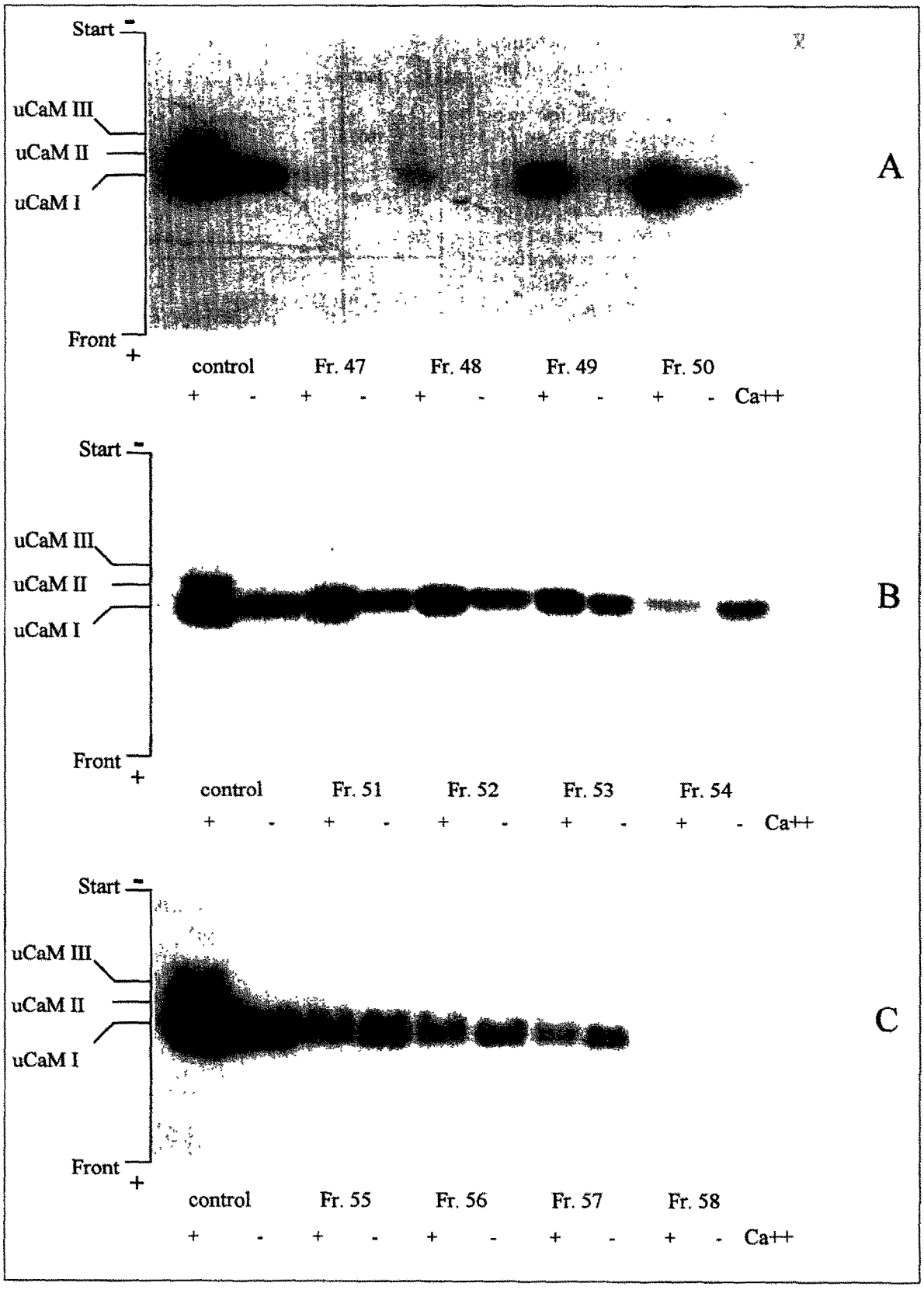
Fig. 1. Separation of two forms of uCaM-synthetase activity by gel filtration of reticulocyte APF II on Superose 6 pg. A sample of 1.8 ml reticulocyte APF II (6 mg/ml) was applied to a column of Superose 6 pg (1 cm i.d. × 48 cm bed height) at 12 ml/h in buffer H at 5°C. Fractions of 0.6 ml were collected and aliquots of 50 μl were tested for activity (incorporation of [^{125}I]ubiquitin into calmodulin, 4 h at 37°) in the FP-test (100 μl). Only those fractions of the chromatogram containing enzymatic activity are shown. For further details see section 2 and the text. (▲), Ca^{2+}-dependent activity; (●), Ca^{2+}-independent activity; (□) $OD_{280\ nm}$ for estimation of protein concentration.

(Ca^{2+} dependence) up to and over 1.0 (Ca^{2+} independence) as the fraction number increases. Obviously two uCaM-synthetase activities displaying different specificities for the two conformations of calmodulin (Ca^{2+}-bound calmodulin, Ca^{2+}-free calmodulin) exist. Therefore in the following experiments these two activities are followed by testing in the presence of 100 μM Ca^{2+} for activity towards Ca^{2+}-bound calmodulin (Ca^{2+} dependence) and in the presence of EGTA for activity towards Ca^{2+}-free calmodulin as substrate (Ca^{2+} independence), respectively. Peak activity is found in fraction 50 for the Ca^{2+}-dependent (670 pmol/h × ml) and in fraction 51 for the Ca^{2+}-independent form (371 pmol/h × ml), respectively. Activity in these peak fractions corresponds to a molecular mass of 230–240 kDa (analytic run not shown), which however in this multi-component system does not necessarily reflect the true molecular mass of the isolated components. Absolute uCaM-synthetase activity decreases approx. 2-fold in passing from the Ca^{2+}-dependent to the fully Ca^{2+}-independent form.

The autoradiograms of the conjugation of calmodulin to [^{125}I]ubiquitin in the individual fractions of the gel filtration experiment of Fig. 1 are shown in Fig. 2. In agreement with the activity measurements and the −Ca^{2+}/+Ca^{2+} activity ratio (Fig. 1) only a very faint band is detectable in fraction 49 in the absence of Ca^{2+}

→

Fig. 2. Autoradiographic analysis of calcium dependence in the fractions obtained by gel filtration on Superose 6 pg. Aliquots of the fractions obtained in the experiment in Fig. 1 were treated and incubated as described for the FP-test in the legend to Fig. 1. The protein in the eluates from the FP-Sepharose of the test was precipitated with TCA and the pellets were neutralized and analyzed by SDS-PAGE (15% gels) as described previously [4,9]. For autoradiography Fuji X-ray film was exposed for 6 days at −80°C and developed as described previously [4,5]. The abbreviations uCaM I-III correspond to the first, second and third order conjugates containing 1–3 ubiquitin molecules/calmodulin, respectively. For further details see legend to Fig. 1, section 2 and the text. (A) Fractions 47–50, +Ca^{2+} and −Ca^{2+}, respectively; (B) fractions 51–54, +Ca^{2+} and −Ca^{2+}, respectively; (C) fractions 55–58, +Ca^{2+} and −Ca^{2+}, respectively. Controls: A–C, lanes 1 and 2 APF II (0.9 mg/ml) +Ca^{2+} and −Ca^{2+}, respectively.


Start
uCaM III
uCaM II
uCaM I
Front
A
control Fr. 47 Fr. 48 Fr. 49 Fr. 50
+ - + - + - + - + - Ca++
Start
uCaM III
uCaM II
uCaM I
Front
B
control Fr. 51 Fr. 52 Fr. 53 Fr. 54
+ - + - + - + - + - Ca++
Start
uCaM III
uCaM II
uCaM I
Front
C
control Fr. 55 Fr. 56 Fr. 57 Fr. 58
+ - + - + - + - + - Ca++

(i.e. + EGTA) whereas from fraction 53 onwards the conjugate synthesized in the absence of Ca^{2+} increases steadily until it is in excess of conjugate generated in the presence of Ca^{2+}.

Since a full separation and isolation of the two uCaM-synthetase activities was not possible by gel filtration procedures, affinity chromatography on ubiquitin Sepharose was attempted. APF IIa was loaded onto ubiquitin Sepharose (not shown) and as can be seen in Table I about 70% of the applied protein runs through the column. The remaining protein is adsorbed and 0.7% can be eluted by DTE, pH 9.0 Buffer F (uCaM-Syn F1). The purification of the two forms of uCaM-synthetase can be followed in Table I from the reticulocyte lysate up to the segregation of uCaM-Syn F1 and uCaM-Syn F2 on ubiquitin Sepharose. The Ca^{2+}-independent activity (activity in the absence of Ca^{2+}) is purified approx. 90-fold fold with a total yield of 2%. The Ca^{2+}-dependent activity (activity in the presence of Ca^{2+}) is enriched approx. 24-fold in one step by ion-exchange chromatography but is then lost during affinity chromatography on ubiquitin-Sepharose. The inactive run-through fraction (uCaM-Syn F2) obtained after chromatography on ubiquitin Sepharose can however be reactivated by the addition of uCaM-Syn F1 (Table I). At a ratio of 1:5 on a protein weight basis of the two pools a specific activity of 975 fkat/mg is obtained, which is approx. 3-fold higher than the specific activity of uCaM-Syn F1.

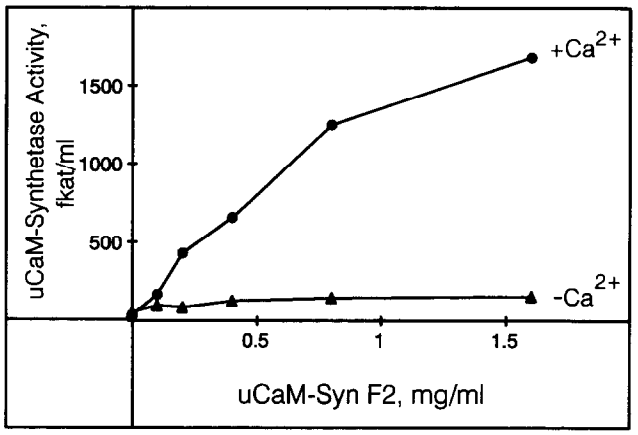


Fig. 3. Stimulation of the uCaM-synthetase activity in uCaM-Syn F1 by addition of the inactive protein uCaM-Syn F2 to the incubation mixture. The incubation mixtures (50 μl) containing 0.1 mg/ml of uCaM-Syn F1 were incubated for 60 min at 37°C with increasing amounts of uCaM-Syn F2 in the FP-test as described in section 2. for further details see Table II, section 2 and the text. (●), 1.1 mM $CaCl_2$ + 1 mM EGTA (+ Ca^{2+}); (▲), 1 mM EGTA ($-Ca^{2+}$)

The activation of uCaM-Syn F1 activity per volume incubation mixture by uCaM-Syn F2 is shown for another preparation in Fig. 3. The basal uCaM-Syn F1 activity of 38 fkat/ml can be increased 44-fold to 1 678 fkat/ml as uCaM-Syn F2 is added to a final concentration of 1.6 mg/ml. Interestingly the Ca^{2+}-independent

Table I

Enrichment and separation of 2 forms of uCaM-synthetase from reticulocytes*

Step	Volume (ml)	Protein (mg/ml)	Specificity activity (fkat/mg)		Purification (*n*-fold)		Activity yield (%)		$-Ca^{2+}$ / $+Ca^{2+}$
			$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$	
1. Reticulocyte lysate	86	35	3.6	24	1	1	100	100	0.2
2. DEAE-Fractogel (APF III)	10	8.0	121	570	33	24	89	63	0.2
3. Passive sorption on Ub-Sepharoze (APE IIa)	20	4.1	113	500	31	24	86	57	0.2
4. Affinity chromatography on Ub-Sepharose									
uCaM-Syn F1 (pH 9-eluate)	1.5	0.4	315	330	89		2		1.0
uCaM-Syn F2 (run-through)	10	5.7	0	0		–		–	–
UCaM-Syn F1 + uCaM-Syn F2			235	975		41		77	0.2

*Each pool was tested in the FP-test (see section 2) where the amount of protein in the total volume of 50 μl was; lysate 320 μg, APF II and APF IIa 45 μg, Ub-Sepharose pH 9.0 eluate 10 μg, Ub-Sepharose run-through 45 μg. In the reconstitution mixture (lowest row) 4.5 μg uCaM-Syn F1 was added to 22.5 μg uCaM-Syn F2 allowing completion of the purification table for Ca^{2+}-dependent activity

Table II

Dependence of uCaM-synthetase activities on the ratio of uCaM-Syn F1 to uCaM-Syn F2*

uCaM-Syn F2	uCaM-synthetase activity							Mixing ratio
	(fkat/ml)		(fkat/ml)		(fkat/mg)		$-Ca^{2+}/+Ca^{2+}$	uCaM-Syn F2
	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$	activity ratio	uCaM-Syn F1 (mg/mg)
	uCaM-Syn F1 0 mg/ml		uCaM-Syn F1 0.1 mg/ml					
0 mg/ml	–		54	38	540	380	1.42	0
0.1 mg/ml	ND		97	168	485	840	0.58	1
0.2 mg/ml	ND		84	430	280	1,433	0.19	2
0.4 mg/ml	ND		126	655	252	1,310	0 19	4
0.8 mg/ml	ND		146	1,248	162	1,386	0.12	8
1.6 mg/ml	0		154	1,678	91	987	0.09	16

*For further details see legend to Fig. 3, section 2 and the text. ND, not determined.

activity also increases approx. 3-fold under these conditions. Since addition of the protein factor uCaM-Syn F2 to the incubation mixture raises the protein concentration, it was checked if the activation of uCaM-Syn F1 also manifests itself as an increase in specific catalytic activity. As demonstrated in Table II the specific activity of uCaM-Synthetase activity does in fact increase nearly 4-fold in the presence of Ca^{2+}. Interestingly the specific activity in the absence of Ca^{2+} (Ca^{2+}-free calmodulin) decreases nearly 6-fold. Thus the $-Ca^{2+}/+Ca^{2+}$ activity ratio decreases 15-fold from 1.42 to 0.09 through the addition of uCaM-Syn F2 to uCam-Syn F1, demonstrating a large specificity shift of enzymatic activity.

4. DISCUSSION

As shown here the Ca^{2+}-dependent ubiquityl-calmodulin synthetase activity is lost during affinity chromatography on ubiquitin Sepharose. Only a Ca^{2+}-independent form of the enzyme is eluted from the affinity gel. This activity was purified 90-fold from reticulocyte lysate with a yield of 2%. The Ca^{2+}-dependent activity could be restored by adding the inactive component found in the run-through of the affinity gel to the Ca^{2+}-independent enzyme fraction (Tables I and II).

Ubiquitin-Sepharose specifically adsorbs enzymes of the E1-, E2-, E3-type [15]. In addition the DTE in the elution buffer can be considered a specific eluent for E1- and E2-type enzymes. Therfore uCaM-Syn F1 as eluted from the ubiquitin-Sepharose under these conditions probably contains a mixture of E1-, E2- and probably E3-type enzymes [15]. The fact that uCaM-Syn F2 does not adsorb to ubiquitin-Sepharose, but is found in the run-through, does not exclude it as an E2-type enzyme [21]. Except that an E1 enzyme is with high probability not contained in this unadsorbed fraction, it is unknown which other components of the E2- and E3-type are present in uCaM-Syn F2 and thus might interact with the components in uCaM-Syn F1. Therefore an interpretation of our results in terms of these component-types is not yet possible.

Enzymatic reactions showing a regulation of catalytic activity by Ca^{2+}/calmodulin can be divided into three different categories according to their $-Ca^{2+}/+Ca^{2+}$ activity ratios: *Type 1*: $-Ca^{2+}/+Ca^{2+}$ activity ratio < 1 (Ca^{2+}-enhanced activity); *Type 2*: $-Ca^{2+}/+Ca^{2+}$ activity ratio = 1 (Ca^{2+}-independent activity) and *Type 3*: $-Ca^{2+}/+Ca^{2+}$ activity ratio > 1 (Ca^{2+}-depressed activity). In a Type 1 reaction Ca^{2+}-bound calmodulin plays the predominant role whereas in a Type 3 reaction Ca^{2+}-free calmodulin is decisive. Both of these reaction types are Ca^{2+}-dependent. Therefore in Ca^{2+}-dependent reactions the direction (activation or inhibition) must be given. In Type 2 reactions neither form surpasses the other.

If these categories are applied to uCaM-synthetase it can be concluded that the enzyme activity normally found in crude extracts or fractions enriched by ion exchange chromatography is of Type 1, i.e. this enzyme primarily recognizes the conformation of Ca^{2+}-bound calmodulin and is therefore activated by Ca^{2+}. In contrast the enzyme eluted from ubiquitin Sepharose (uCaM-Syn F1) is of Type 2 with a tendency towards Type 3 (Ca^{2+}-independent, Ca^{2+}-depressed activity). A mixing of the inactive protein uCaM-Syn F2 with the catalytically active fraction uCaM-Syn F1 results in a Type 1 enzyme again. The transition from the reaction Type 2–3 to Type 1 as demonstrated in Table II is reflected in a dramatic 15-fold change in the $-Ca^{2+}/+Ca^{2+}$ activity ratio indicative of a corresponding specificity change of enzymatic activity.

One plausible explanation of the experiments can be

expressed in a model in which uCaM-Syn F1 corresponds to a catalytic component (one catalytic site) which can be modulated by a regulatory component in uCaM-Syn F2. The regulatory component which has no catalytic activity not only enhances the activity of uCaM-synthetase in uCaM-Syn F1 44-fold but also appears to alter the substrate specificity from the Ca^{2+}-free form of calmodulin towards the Ca^{2+}-bound form of calmodulin. The model could also explain the 3-fold increase in Ca^{2+}-independent activity (Table II) by assuming that the specificity change is not perfect allowing the residual Ca^{2+}-independent activity a limited increase.

In another model one could also envision two different enzymes (i.e. two catalytic centers). One enzyme is specific for Ca^{2+}-free calmodulin and is already active in uCaM-Syn F1. The other enzyme is specific for Ca^{2+}-bound calmodulin but inactive in uCaM Syn F2 and active when mixed with uCaM-Syn F1. Addition of uCaM-Syn-F 2 to uCaM-Syn F1 would lead to an activation of the latent Ca^{2+}-dependent enzyme in uCaM-Syn F2. However, in this model the Ca^{2+}-independent activity should remain constant under these conditions.

From the data it can be concluded that uCaM-Syn F1 is probably saturated with uCaM-Syn F2 in reticulocytes so that the Ca^{2+}-dependent form of uCaM-synthetase (Type 1) is the biologically relevant one (see Tables I and II).

REFERENCES

[1] Gehrke, P.P. and Jennissen, H.P. (1987) Biol. Chem. Hoppe-Seyler 368, 691–708.
[2] Gehrke, P., Ziegenhagen, R.G. and Jennissen, H.P. (1987) Biol. Chem. Hoppe-Seyler 368, 744.
[3] Ziegenhagen, R., Gehrke, P.P. and Jennissen, H.P. (1988) FEBS Lett. 237, 103–107.
[4] Ziegenhagen, R. and Jennissen, H.P. (1988) Biol. Chem. Hoppe-Seyler 369, 1317–1324.
[5] Jennissen, H.P. and Laub, M. (1988) Biol. Chem. Hoppe-Seyler 369, 1325–1330.
[6] Ziegenhagen, R., Goldberg, M., Rakutt, W.-D. and Jennissen, H.P. (1990) FEBS Lett. 271, 71–75.
[7] Laub, M. and Jennissen, H.P. (1991) FEBS Lett. 294, 229–233.
[8] Ziegenhagen, R. and Jennissen, H.P. (1990) FEBS Lett. 273, 253–256.
[9] Jennissen, H.P., Botzet, G., Majetschak, M., Laub, M., Ziegenhagen, R. and Demiroglou, A. (1992) FEBS Lett., 296, 51–56.
[10] Laub, M. and Jennissen, H.P. (1989) Biol. Chem. Hoppe-Seyler 370, 926–927.
[11] Gilbert W., Marchionni M. and McKnight G. (1986) Cell 46, 151–154.
[12] Sogin M.L., Elwood, H.J. and Gunderson J.H. (1986) Proc. Natl. Acad. Sci. USA 83, 1383–1387.
[13] Majetschak, M., Laub, M. and Jennissen, H.P. (1992) Biol. Chem. Hoppe-Seyler 373, 797–798.
[14] Ciechanover, A., Hod., Y. and Hershko, A. (1978) Biochem. Biophys. Res. Commun. 81, 1100–1105.
[15] Hershko, A, Heller, H., Eytan, E. and Reiss, Y. (1986) J. Biol. Chem. 261, 11992–11999.
[16] Ciechanover, A., Heller, H., Elias, S., Haas, A.L. and Hershko, A. (1980) Proc. Natl. Acad. Sci. USA 77, 1365–1368.
[17] Autric, F., Ferraz, C. Kilhofer, M.-C., Cavadore, J.-C. and Demaille, J.G. (1980) Biochim. Biophys. Acta 631, 139–147.
[18] Rochette-Egly, C., Boschetti, E., Basset, P. and Egly, J.-M. (1982) J. Chromatogr. 241, 333–344.
[19] Laemmli, K.K. (1970) Nature 227, 680–685.
[20] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[21] Klemperer, N.S., Berleth, E.S. and Pickart, C.M. (1989) Biochemistry 28, 6035–6041.