Calmodulin carboxylmethyl ester formation in intact human red cells and modulation of this reaction by divalent cations in vitro

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Calmodulin *Protein–glutamic(aspartic)-methyltransferase* *Erythrocyte* *Protein S-100* *Cation*

1. INTRODUCTION

While the formation of carboxylmethyl ester of membrane proteins correlates well with functional changes (chemotaxis) in bacteria [1–3], almost nothing is known about a functional role of this reaction in mammalian systems. On the other hand, the formation of carboxylmethyl esters can be shown for soluble as well as membrane proteins in a variety of mammalian systems (review [4]). One problem arises from the fact that purified protein–glutamic(aspartic)-methyltransferase (see footnote) from bovine brain catalyzes the in vitro carboxylmethylester formation of many proteins, but there is no proof that these proteins are carboxylmethylated in the living cell.

For this reason it seemed very important when it was reported [5] that the regulatory protein calmodulin could be carboxylmethylated not only in vitro but also in living cells. Walker ascites carcinoma cells were incubated with L-[*methyl*-^{3}H]-methionine, purified calmodulin by affinity chromatography, and found that also under these conditions calmodulin contained carboxylmethyl esters.

Following a proposal of the NC–IUB we use the terminus protein–glutamic(aspartic)-methyltransferase instead of the former *S*-adenosyl-L-methionine:protein *O*-methyltransferase or methyltransferase II (EC 2.1.1.24)

For mere reasons of readability we use the expression 'carboxylmethylated protein' instead of the more exact formulation 'glutamic(aspartic)methylester-containing protein'

However, we found that extracts from tissues which had been incubated with [*methyl*-^{3}H]-methionine prior to homogenization continued to transfer methylgroups to protein carboxyl groups. Under these circumstances it is difficult to exclude the possibility that protein-carboxylmethylation of a given protein had occurred only after destruction of the cells.

Therefore, we have examined the problem of intracellular carboxylmethylation of calmodulin in intact human erythrocytes under conditions where an incorporation of methyl groups after destruction of the cells was excluded. According to the results obtained, calmodulin undergoes protein-carboxylmethyl ester formation in the intact cell.

We will show furthermore that the degree of protein-carboxylmethyl ester formation was affected by the nature of divalent cations present.

2. METHODS AND MATERIALS

2.1. *Preparation and incubation of human red cells*

Fresh heparinized human blood (30 ml) was spun for 5 min at 4000 × g_{max}. The plasma was discarded by the pellet washed 3 times with phosphate-buffered saline (0.17 M NaCl, 5 mM sodium phosphate (pH 7.2)). To 12 ml sedimented erythrocytes, 20 ml incubation medium (sodium phosphate 125 mM (pH 7.2); $MgCl_2$ 12.5 mM; ATP 5 mM; glucose 11 mM) was added and the pH adjusted to pH 7.2. The reaction was started by addition of 1 mCi L-[*methyl*-^{3}H]methionine (spec. act. 15 Ci/mmol) in 1 ml H_2O. After mixing, the sample was incubated for 3 h at 37°C in a shaking water bath (shaking frequency ~ 50/min). The

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incubation was stopped by cooling in ice, and the erythrocytes were sedimented by centrifugation at 4000 × g_{max} for 10 min. The supernatant was carefully removed. The sedimented erythrocytes were immediately lysed by addition of 70 ml ice-cold lysing solution. The lysing solution had been prepared by dissolving 52 μmol *S*-adenosylhomocysteine in 2 ml 3% acetic acid, followed by dilution with 150 ml ice-cold H_2O. To this, 13 mg phenylmethylsulfonylfluoride dissolved in 1 ml methanol was added.

It was imporant that the following steps were performed at pH < 6.5. After lysis, the mixture was spun for 20 min at 40 000 × *g*, the supernatant decanted and the sediment again suspended in the lysing solution followed by another centrifugation at 40 000 × g for 20 min. The supernatant was combined with the first supernatant, and 0.8 g DEAE-cellulose (Cellex-D) was added. DEAE-cellulose chromatography was continued as in [6] except that the pH was always kept at pH 6.3. About half of the eluted calmodulin was used for affinity chromatography (see below). The rest was treated with 2.5 g solid trichloroacetic acid at 0°C. Precipitated proteins were sedimented by centrifugation at 40 000 × *g* for 20 min. To the pellet 120 μl 10% (w/v) sodium dodecylsulfate (SDS), 10 μl 2-mercaptoethanol and 20 μl 1 M sodium phosphate (pH 6.0) were added, the pellet resuspended and incubated for 15 min at 56°C. Of this suspension 20–30 μl were used for electrophoresis.

2.2. *Affinity chromatography*

Fluphenazine was coupled to epoxy-activated Sepharose 6B according to [7]. Fluphenazine–Sepharose (1 ml) was filled into a small column (0.75 × 2.5 cm) and equilibrated with 15 mM imidazole–HCl (pH 6.3), containing 0.6 M NaCl, 5 mM 2-mercaptoethanol and 1 mM $CaCl_2$. The calmodulin containing fraction from the DEAE-cellulose step which had been brought to 5 mM 2-mercaptoethanol and 1 mM $CaCl_2$ was loaded onto the column. The column was washed with 5 ml of the equilibration medium. To the effluent collected during these steps (20 ml) 3.5 g solid trichloroacetic acid was added while the sample was cooled at 0°C. The precipitated proteins were sedimented by centrifugation at 40 000 × *g* for 20 min. The pellet was dissolved in buffered SDS for electrophoresis as given above.

Calmodulin was eluted from the column with 15 mM imidiazole–HCl (pH 6.3), containing 0.6 M NaCl, 5 mM 2-mercaptoethanol, and 2 mM EGTA. To the first 10 ml effluent 1 mg ovalbumin was added, and the proteins were precipitated by addition of 2 g solid trichloroacetic acid. Centrifugation of the precipitated proteins and treatment with buffered SDS was carried out as given above.

Electrophoresis was carried out in 9% polyacrylamide round gels (0.5 × 12 cm) in 0.1 M sodium phosphate (pH 6.0) containing 0.2% SDS with 0.05% Fast Green FCF in 87% glycerol as front marker. Gels were stained for protein with 0.05% Coomassie brillant blue G-250.

The gels were cut into 4 mm long discs, which were transferred to liquid scintillation vials, dissolved at 50°C in 1 ml 30% H_2O_2, and counted with 12 ml Unisolve after chemiluminescence had decayed.

2.3. *In vitro protein-carboxylmethyl ester formation with calmodulin and purified protein–glutamic(aspartic)-methyltransferase*

In vitro carboxylmethylation was performed with calmodulin from bovine brain and testes. In vitro carboxylmethylation in the presense of different divalent cations was performed with calmodulin purified from bovine testes. The control incubation contained (final conc.): calmodulin 100 μM; *S*-adenosyl-L-[*methyl*-^{3}H]methionine 200 μM (spec. act. 0.5 Ci/mmol); sodium phosphate 100 mM (pH 6.0); EGTA 1 mM; purified protein–glutamic(aspartic)-methyltransferase from calf brain 60 U/ml. When the effect of divalent cations was measured, the incubation contained in addition 3 mM $CaCl_2$, $MgCl_2$, or $MnCl_2$.

When the effect of calcium on the spontaneous hydrolysis of protein–carboxylmethyl esters of calmodulin was measured, calmodulin was first carboxylmethylated in the presence or absence of exogenous calcium for 30 min as above. The further incorporation of methyl-groups was then stopped by addition of 3 mM *S*-adenosylhomocysteine and the amount of protein–carboxylmethyl esters was followed for another 120 min.

2.4. *Materials*

Epoxy-activated Sepharose 6B was from Pharmacia (Freiburg); Cellex-D DEAE-cellulose from

Bio-Rad (Munich); Unisolve from Zinsser (Frankfurt); L-[*methyl*-^{3}H]methionine and *S*-adenosyl-L-[*methyl*-^{3}H]methionine from Amersham-Buchler (Braunschweig); *S*-adenosyl-homocysteine was purchased from Serva (Heidelberg); fluphenazine–2 HCl was a generous gift of Dr M. Rohmer (Chemische Fabrik Von Heyden, Regensburg).

Protein–glutamic(aspartic)-methyltransferase was purified from calf brain as in [8] and spec. act. 10 000 U/mg (1 unit ± 1 pmol protein-carboxylmethyl ester formed/min at 37°C with ovalbumin as substrate). Calmodulin was purified either from bovine brain as in [9] or from bovine testes as in [10]. The preparation from bovine brain still contained some S-100 protein whereas the preparation from bovine testes was pure according to gel electrophoretic criteria.

3. RESULTS

3.1. *Experiments with human erythrocytes*

When human erythrocytes pre-incubated in the presence of L-[*methyl*-^{3}H]methionine, were lysed in the presence of *S*-adenosylmethionine, >80% of incorporated radioactivity was found in the membrane fraction obtained after centrifugation of the lysate (~ 10^6 dpm/mg protein). The incorporated radioactivity represented to ~ 80% protein–carboxylmethyl esters. The lysate which contained ~ 20% of incorporated radioactivity was fractionated by DEAE-cellulose chromatography and the calmodulin-containing fractions were examined by SDS electrophoresis and autoradiography. As depicted in fig.1a, radioactivity appeared in various bands, the sharpest and biggest peak being located at the position of calmodulin. Passage of the DEAE-purified fraction over fluphenazine–Sepharose removed almost completely the protein and the radioactivity corresponding to calmodulin (fig.1b). In the presence of EGTA, highly purified calmodulin eluted from the affinity column together with ^{3}H radioactivity (fig.1c); 90% of the radioactivity was alkaline labile, could be extracted into toluene–isoamylalcohol, and removed from there by evaporation at 80°C, indicating that the radioactivity represented protein-carboxylmethyl esters and not *N*-methylated lysyl residues. These results provide evidence that calmodulin is a substrate for protein–glutamic(aspartic)-methyltransferase in the intact human erythrocyte and

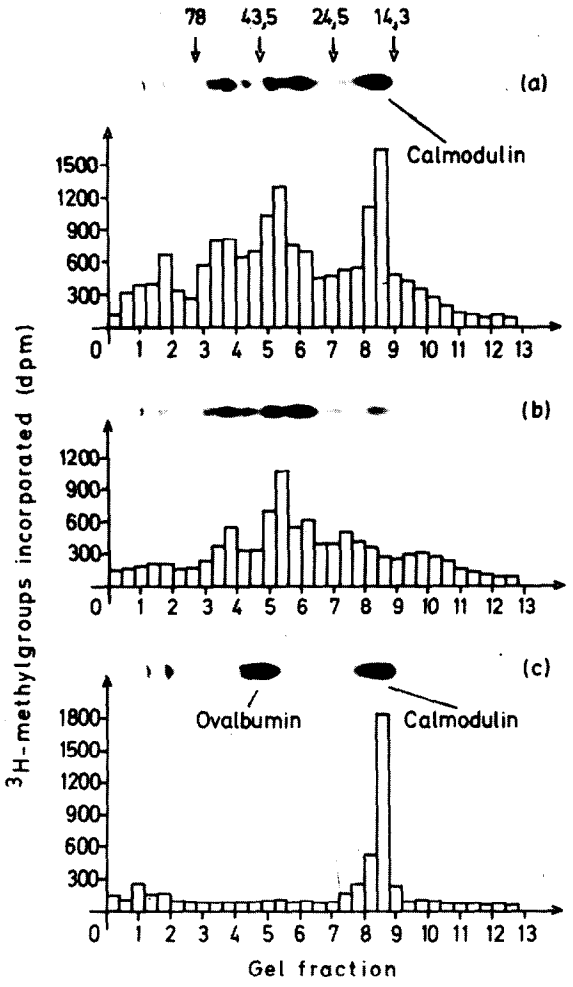

Fig.1. Protein stains and distribution of radioactivity of polyacrylamide–SDS electrophoretograms of protein fractions isolated from human erythrocytes after incubation with L-[*methyl*-^{3}H]methionine: (a) calmodulin-containing fraction after DEAE-cellulose chromatography. The numbers on top of the Coomassie blue stain represent the position of the following marker enzymes (M_r × 10^{-3}): conalbumin (78); ovalbumin (43.5); trypsinogen (24.5); lysozyme (14.5); (b) protein mixture in (a) after passage over a fluphenazine–Sepharose column; (c) protein fraction eluted from the fluphenazine–Sepharose column with an EGTA-containing buffer (section 2). Ovalbumin was added to the eluate to allow for a complete precipitation of the proteins by trichloroacetic acid prior to electrophoresis. For details of the incubation of erythrocytes, and the purification and electrophoresis of calmodulin under low-pH conditions see section 2.

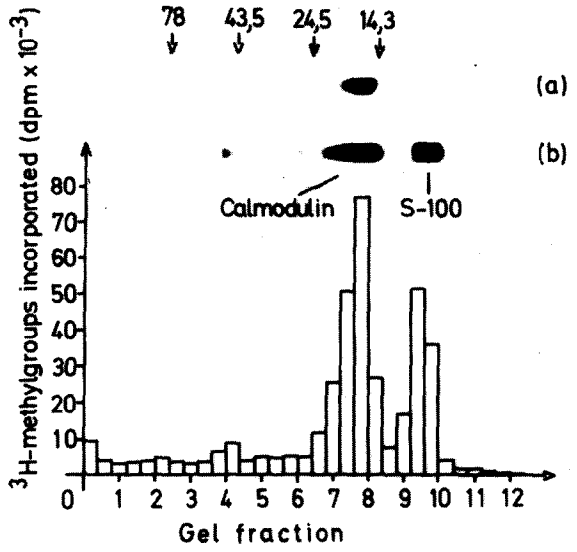

Fig.2. In vitro protein–carboxylmethyl ester formation with calmodulin and S-100 protein from bovine brain. The incubation was done for 60 min at 37°C with 0.3 mg substrate protein, 10 μM *S*-adenosyl-L-[*methyl*-^{3}H]-methionine (spec. act. 15 Ci/mmol), and 50 U/ml of protein–glutamic(aspartic)-methyltransferase. For further details see section 2. The upper part of the figure shows Coomassie blue stains of calmodulin from bovine testes purified by affinity chromatography (a) and of calmodulin and protein S-100 from bovine brain (b). The lower part of the panel shows the incorporation of [^{3}H]methyl-groups into (b). The numbers and arrows on top of (a) represent the position of marker enzymes (see fig.1 legend). The radioactivity incorporated into the two protein fractions was alkaline-labile, could be extracted into isoamylalcohol–toluene, and was evaporated therefrom by heating at 80°C.

that calmodulin in the intact cell exists at least in part in the carboxylmethylated state.

Erythrocytes incubated with L-[*methyl*-^{3}H]-methionine which had been lysed in the absence of exogenous *S*-adenosylhomocysteine showed at 35% higher total protein-carboxyl[^{3}H]methyl ester formation than in the presence of 0.3 mM *S*-adenosylhomocysteine indicating the importance of the addition of this inhibitor to avoid the 'unphysiological' formation of protein-carboxylmethyl esters.

Table 1

Substrate specificity of protein-carboxylmethyl ester formation catalyzed by calf brain protein–glutamic(aspartic)-methyltransferase

Substrate	Carboxylmethylester formed (pmol . mg protein^{-1} . h^{-1})
Calmodulin (bovine testes)	2345
Depleted ghosts (human erythrocytes)	780
Ovalbumin	365
Trypsinogen (bovine pancreas)	80
Ribonuclease A (bovine pancreas)	55
Trypsin (bovine pancreas)	30
Chymotrypsinogen A (bovine pancreas)	30

Substrate protein (1 mg) was incubated for 60 min at 37°C in a test volume of 200 μl containing (final conc.): Sodium phosphate, 100 mM (pH 6.0); *S*-adenosyl-L-[*methyl*-^{3}H]methionine (spec.act.) 2 Ci/mmol), 10 μM; protein–glutamic(aspartic)-methyltransferase, 50 U/ml

3.2. *In vitro experiments with purified protein–glutamic(aspartic)-methyltransferase*

Calmodulin from bovine brain purified as in [9] showed two radioactive peaks in the polyacrylamide electrophoretogram after in vitro incubation with *S*-adenosyl[^{3}H]methionine and purified protein–glutamic(aspartic)methyltransferase (fig.2), the main peak corresponding to calmodulin, the second peak with higher app. M_r-value to the position of the S-100 protein, which is known to be frequently a contaminant of preparations from brain [9].

Calmodulin purified by affinity chromatography from bovine testes showed only one protein band (fig.2) and only the radioactivity peak corresponding to calmodulin (not shown). Protein-carboxylmethyl ester formation with calmodulin from bovine testes was performed in vitro and compared with other substrates for protein–glutamic(aspartic)-methyltransferase. The results are given in table 1. Of all substrates tested, calmod-

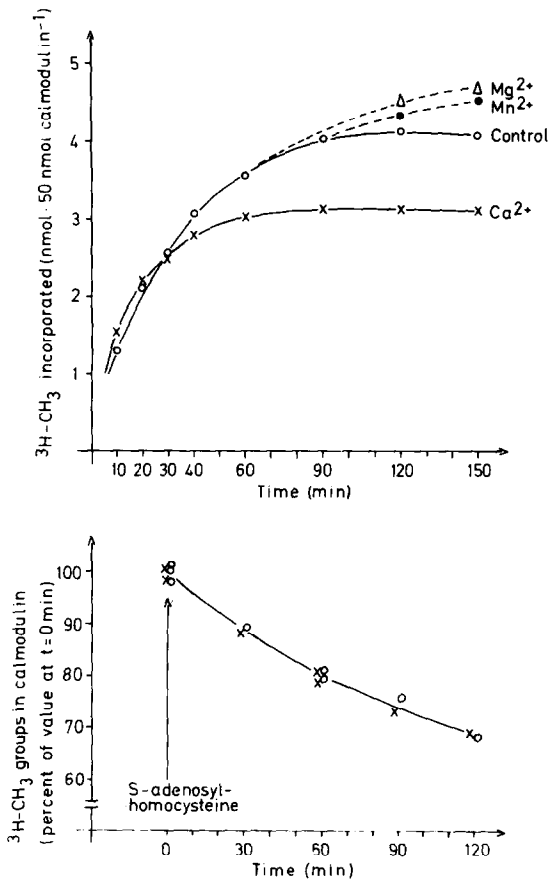

Fig.3. Effects of divalent cations on protein-carboxylmethyl ester formation with purified calmodulin and on methyl-ester hydrolysis with carboxylmethylated calmodulin. Upper: Calmodulin purified from bovine testes was incubated with protein–glutamic(aspartic)-methyltransferase and *S*-adenosyl-L-[*methyl*-^{3}H]-methionine in the presence or absence of divalent cations as given in the text. All incubations contained 1 mM EGTA. To the control no divalent cations were added. The other incubations contained (total conc.) 3 mM $CaCl_2$, 3 mM $MnCl_2$, or 3 mM $MgCl_2$. Lower: Calmodulin was carboxylmethylated in the presence of 1 mM EGTA with (+ —— +) or without (o——o) 3 mM $CaCl_2$. After 30 min the formation of protein-carboxyl methyl esters was stopped by addition of *S*-adenosyl homocysteine (final conc. 3 mM), and the hydrolysis of protein carboxylmethyl esters was followed for another 2 h at 37°C.

ulin was by far the best acceptor protein (on a mg protein basis).

3.3. *Effects of divalent cations*

When purified calmodulin from bovine testes was incubated with protein–glutamic(aspartic)-methyltransferase in the presence of EGTA without addition of exogenous divalent cations, a maximum of 82 nmol (CH_3)/μmol calmodulin was incorporated within 120 min (fig.3a).

In the presence of calcium, however, ≤62 nmol (CH_3)/μmol were incorporated over 120 min. With Mn^{2+} or Mg^{2+} the incorporation of methylgroups proceeded during the first 90 min almost in the same way as in the absence of exogenous divalent cations, but the incorporation continued for >150 min whereas in the presence of calcium or the absence of exogenous divalent cations a saturating level was reached after 90–120 min. As we shall show elsewhere (in preparation), the 'saturation' does not result from a piling up to inhibitory concentrations of *S*-adenosylhomocysteine, but rather represents a steady state where incorporation equals spontaneous methyl ester hydrolysis. We therefore considered the possibility that the lower steady-state protein-carboxylmethyl ester formation in the presence of calcium resulted from a higher rate of spontaneous demethylation. As shown in fig.3b, this was not the case. The rate of spontaneous demethylation was the same in the presence or absence of calcium.

4. DISCUSSION

Our results obtained with human erythrocytes show that carboxylmethyl ester formation of calmodulin occurs in a living cell. This is in agreement with the finding in Walker ascites carcinoma cells [5]. By introducing *S*-adenosylhomocysteine to the lysing medium, we could exclude that calmodulin had become carboxylmethylated after disrupting the cells.

In view of the fact that most mammalian cells contain protein–glutamic(aspartic)-methyltransferases, it seems likely that in most cells calmodulin exists at least in part in the carboxylmethylated state. The high methyl-group accepting capacity of calmodulin found in vitro is not surprising as calmodulin from bovine brain contains 27 glutamic and 23 aspartic residues/molecule [11]. For the same reason the S-100 protein is also a good substrate for protein–glutamic(aspartic)-methyltransferase (fig.2), but it still

has to be proven whether this protein exists also in the carboxylmethylated form in the intact brain. At this point, it should be noted that proteins serving as substrates for protein–glutamic(aspartic)-methyltransferase in vitro must not necessarily exist in a carboxylmethylated form in vivo. Although trypsinogen is an excellent substrate for protein–glutamic(aspartic)-methyltransferase in vitro, we could not find carboxylmethyl ester formation with trypsinogen in isolated pancreatic lobules under conditions of high rates of trypsinogen synthesis (unpublished).

Calcium clearly affects the steady-state degree of protein-carboxylmethyl ester formation with calmodulin. It could:

(1) Affect the conformation of calmodulin and by doing so render some acid residues on the molecule less accessible to protein–glutamic(aspartic)-methyltransferase;
(2) Favor spontaneous demethylation.

As we could not find an effect of calcium on the rate of spontaneous demethylation it seems likely that the first explanation is correct. According to [12], free calmodulin contains 28% α-helical structure. The percentage of α-helical structure increases to 39%, 40% and 42% in the presence of Mg^{2+}, Mn^{2+} and Ca^{2+}, respectively. If the degree of α-helical structure would be directly correlated with the steady-state degree of carboxylmethylation, one would expect the curves with Mn^{2+} and Mg^{2+} to lie between the calcium curve and the control curve in fig.3a. The fact that this was not the case indicates that other structural parameters are responsible for the differences observed. The steady-state degree of carboxylmethylation might be a useful parameter in the study of conformational changes of calmodulin.

The main question is whether protein-carboxylmethyl ester formation affects the binding of calcium to calmodulin and/or the binding of calmodulin to specific proteins. Experiments concerning this question are hampered by the fact that it is very difficult to achieve in vitro a state in which at least 1 protein-carboxylmethyl ester is formed/calmodulin molecule. In [5], calmodulin pre-carboxylmethylated in vitro to ~ 50% was less potent in activating phosphodiesterase. For the future it seems important to study not only the effect of carboxylmethylation of calmodulin on its various properties, but also the steady-state degree of carboxylmethylation of calmodulin in living cells.

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