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# Methylation of calmodulin at carboxylic acid residues in erythrocytes

## A non-regulatory covalent modification?

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The physiological role of protein carboxy-group methylation reactions in human erythrocytes was studied with calmodulin as an endogenous methyl-group acceptor. The steady-state degree of calmodulin carboxy-group methylation is substoichiometric both in intact cells and in a lysed-cell system (about 0.0003 mol of methyl groups/mol of polypeptide). Purified erythrocyte calmodulin is a substrate for a partially purified erythrocyte carboxy-group methyltransferase and can be methylated to the extent of about 0.0007–0.001 mol of methyl groups/mol of polypeptide. This erythrocyte protein methyltransferase displays an apparent specificity for atypical racemized and/or isomerized D-aspartate and L-isoaspartate residues [McFadden & Clarke (1982) Proc. Natl. Acad. Sci. U.S.A. **79**, 2460–2464; Murray & Clarke (1984) J. Biol. Chem. **259**, 10722–10732]. Exposure of calmodulin to elevated temperatures before methylation results in racemization of aspartate and/or asparagine residues, and may result in isoaspartate formation as well. The methylatability of these samples also increases as a function of time of heating, independent of the pH (over the range pH 5–9) or $Ca^{2+}$ concentration; the most significant increase occurs during the initial 60 min, when calmodulin retains a fraction of its biological activity. These results are consistent with the hypothesis that methylation of calmodulin may occur at these uncommon aspartate residues, but are not consistent with a regulatory role for the methylation reaction.

## INTRODUCTION

Calmodulin is the ubiquitous $Ca^{2+}$-binding protein that has been shown to activate many enzymes in a $Ca^{2+}$-dependent manner (for reviews see Cheung, 1980; Klee & Vanaman, 1982; Manalan & Klee, 1984). Calmodulin is a small highly acidic protein that usually contains a modified amino acid, trimethyl-lysine, at position 115 (Jackson *et al.*, 1977). There has been considerable interest in the possibility that calmodulin may also be methylated at carboxylic acid residues. Calmodulin, isolated from bovine brain or testes, is a good methyl-group acceptor for protein methyltransferase activities from erythrocytes and brain (Kloog *et al.*, 1980; Gagnon *et al.*, 1981; Runte *et al.*, 1982; Billingsley *et al.*, 1983, 1984; Murtaugh *et al.*, 1983; Johnson *et al.*, 1985). There is, however, some question as to the stoichiometry of this methylation reaction. Although there is one report of the incorporation of up to 0.50 mol of methyl groups/mol of bovine brain calmodulin *in vitro* (Gagnon *et al.*, 1981), other investigators have reported much lower stoichiometry of methylation. For calmodulin from bovine testes, a maximal methyl-group incorporation *in vitro* of 8.2% was observed in the presence of EGTA, the value being dependent on the presence or absence of various bivalent cations (Runte *et al.*, 1982). Methyl-group incorporation into purified bovine brain calmodulin was found to be less than 5%, independent of the concentration of $Ca^{2+}$, in one study (Billingsley *et al.*, 1984), and 1–2% in another (Johnson *et al.*, 1985).

The physiological significance of this methylation reaction is not presently clear. Conflicting evidence has been presented for the proposal that the carboxy-group methylation of calmodulin may modulate its ability to activate various target enzymes (Gagnon *et al.*, 1981; Billingsley *et al.*, 1983, 1984). On the other hand, it has been proposed that protein methyltransferases from bovine brain and human erythrocytes specifically recognize racemized and isomerized aspartate residues as the first step in the metabolism of altered proteins (McFadden & Clarke, 1982; O'Connor *et al.*, 1984; Clarke, 1985). Such a reaction would then only recognize the subpopulation of calmodulin molecules that contain D-aspartate and/or L-isoaspartate residues (McFadden & Clarke, 1982; Aswad, 1984*b*; Murray & Clarke, 1984). In this regard, it has been proposed that L-isoaspartate residues resulting from asparagine deamidation reactions represent the predominant methyl-group-acceptor sites in bovine brain calmodulin (Johnson *et al.*, 1985).

In light of these studies, we were interested in determining the steady-state degree of calmodulin carboxy-group methylation in human erythrocytes both in intact cells *in vivo* and in a lysed-cell system *in vitro*. Runte *et al.* (1982) have reported that carboxy-group methylation of calmodulin occurs in erythrocytes incubated under conditions designed to reflect those pertaining *in vivo*. We now show here that, although calmodulin is methylated in intact cells, the extent of this methylation is so low that it is hard to envision a regulatory role for this modification. Pretreatment of

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calmodulin, under conditions that would be expected to increase the content of racemized or isomerized amino acids, dramatically increased its ability to act as a methyl-group acceptor.

## MATERIALS AND METHODS

### Materials

*S*-Adenosyl-L-homocysteine, DEAE-Sephadex (A-25), imidazole, bovine pancreatic ribonuclease A and reagents for the $(Ca^{2+}+Mg^{2+})$-stimulated ATPase and $P_i$ assays were obtained from Sigma Chemical Co. Spectropor 3 dialysis tubing ($M_r$ cut-off approx. 3500) was obtained from Spectrum Medical Industries. Affi-Gel Phenothiazine (phenothiazine coupled to a cross-linked agarose) was generously given by Dr. Sheldon Engelhorn, Bio-Rad Laboratories. NCS tissue solubilizer, ACS II and OCS scintillation cocktails, as well as *S*-adenosyl-L-[*Me*-$^3$H]methionine (15 Ci/mmol; 1 mCi/ml), and L-[*Me*-$^3$H]methionine (72 Ci/mmol; 1 mCi/ml) were obtained from Amersham–Searle. *S*-Adenosyl-L-methionine (hydrogen sulphate salt, 98% pure) was purchased from Boehringer–Mannheim. All other chemicals were analytical-reagent grade.

### Erythrocyte preparation

Human blood was drawn from volunteer donors by venepuncture into heparinized tubes. Whole blood was centrifuged at 5000 *g* for 5 min at 0–4 °C; the plasma and buffy coat were removed by aspiration. The cell pellet was then washed three times in 10–20 vol. of phosphate-buffered saline (150 mM-NaCl/5 mM-sodium phosphate buffer, pH 7.4) at 0–4 °C followed by a final wash in iso-osmotic Tris/HCl (172 mM-Tris/HCl buffer, pH 7.4). For cells used in the experiments studying methylation *in vivo* and *in vitro*, phosphate-buffered saline supplemented with 0.3% glucose was used for the final wash.

### Purification of erythrocyte calmodulin

Calmodulin was purified from human erythrocytes as outlined below. All manipulations were done at 0–4 °C.

**Preparation of cell lysate.** Recently outdated blood was obtained from the University of California at Los Angeles blood bank; the cells were then washed as described above. The washed packed cells were lysed in 14 vol. of 1 mM-disodium EDTA, pH 6.0. The membranes were removed from the lysate by centrifugation at 23000 *g* for 10 min. The membrane pellet was reserved for use in the calmodulin assay, as described below.

**DEAE-Sephadex chromatography.** Calmodulin was partially purified from erythrocyte lysate by DEAE-Sephadex chromatography by the method of Jarrett & Kyte (1979). In order to minimize the loss of methyl esters, the pH of the eluting buffer was lowered to 6.3. Fractions were collected and were analysed for calmodulin activity by the activation of the erythrocyte membrane $(Ca^{2+}+Mg^{2+})$-stimulated ATPase in the presence of $Ca^{2+}$. In addition, a portion of each fraction was subjected to SDS/polyacrylamide-gel electrophoresis with the system of Laemmli (1970). The fractions containing calmodulin [i.e. the fractions that contain a 17000-$M_r$ protein as shown by gel electrophoresis and that activate the $(Ca^{2+}+Mg^{2+})$-stimulated ATPase in a $Ca^{2+}$-dependent manner] were pooled and dialysed against 40 vol. of doubly distilled water in Spectropor 3 dialysis tubing. After two buffer changes the material was freeze-dried. (The dialysis and freeze-drying steps were included to concentrate the sample when dealing with large-scale calmodulin purifications from outdated blood. These steps were omitted when [$^3$H]methylated calmodulin was to be isolated from fresh erythrocytes methylated *in vivo* or *in vitro*.)

The column size used for the large-scale preparation of calmodulin (e.g. from 5 litres of membrane-free lysate) was approx. 2.7 cm diam. × 5.5 cm long. The column size was accordingly reduced for smaller-scale purifications (i.e. isolation of calmodulin that had been methylated *in vivo* and *in vitro*).

**Affinity chromatography.** Calmodulin from the DEAE-Sephadex purification was further purified by affinity chromatography by modification of the method of Jamieson & Vanaman (1979). This technique takes advantage of the fact that calmodulin will bind to phenothiazine in a reversible $Ca^{2+}$-dependent manner. Briefly, the freeze-dried DEAE-Sephadex-purified calmodulin sample was suspended in a minimum volume of calcium buffer (600 mM-NaCl/5 mM-$CaCl_2$/1 mM-2-mercaptoethanol/30 mM-imidazole/HCl buffer, pH 6.3). Particulate matter was removed by centrifugation at 5000 *g* for 10 min. The supernatant was adsorbed on a column of Affi-Gel Phenothiazine that had been previously equilibrated with calcium buffer. The column size used for large-scale purifications (e.g. from 5 litres of lysate) was approx. 1.5 cm × 10 cm. The column size was reduced for smaller-scale purifications. The column was then washed with the calcium buffer, at a flow rate of 8 ml/h, until the $A_{280}$ of the eluate was less than 0.100. Calmodulin was then eluted from the column with an EGTA elution buffer (5 mM-EGTA/1 mM-2-mercaptoethanol/30 mM-imidazole/HCl buffer, pH 6.3). In order to stabilize the calmodulin, the EGTA in each fraction collected was 'neutralized' by the addition of an equimolar amount of 1 M-$CaCl_2$. Each fraction was analysed by gel electrophoresis, and, in addition, portions of each fraction were assayed for calmodulin activity as described below. The fractions containing calmodulin were pooled, dialysed against 40 vol. of 1 mM-$CaCl_2$ (one buffer change), and finally dialysed against 40 vol. of doubly distilled water (two buffer changes), and freeze-dried. (The dialysis and freeze-drying steps were omitted when [$^3$H]methylated calmodulin was to be isolated from fresh erythrocytes methylated *in vivo* or *in vitro*.)

### Assay of calmodulin activity

Calmodulin was assayed by the method of Jarrett & Kyte (1979). This assay procedure measures the calmodulin-mediated $Ca^{2+}$-dependent activation of the $(Ca^{2+}+Mg^{2+})$-stimulated ATPase present in erythrocyte membranes. Erythrocyte membranes were prepared as described by the authors cited and were stored at −70 °C until needed. These membranes retained their $(Ca^{2+}+Mg^{2+})$-stimulated ATPase activity for up to 6 months. $P_i$ released from ATP during the ATPase assay was determined by the method of Ohnishi & Gall (1978). Analyses were carried out in triplicate, with 0.15 mg of membrane protein per assay tube. The amount of calmodulin used in each assay tube ranged from 5 to 5000 ng. The amount of calmodulin required to give 50%

of the maximal stimulation of the ATPase (1 unit of calmodulin) was found to be approx. 30–60 ng, and the maximum stimulation was typically 1.5–2.5-fold.

### Methylation of calmodulin in intact cells

L-[*Me*-$^3$H]Methionine (0.0625 μmol; 72 Ci/mmol) was freeze-dried in a polyethylene bottle containing 1.56 μmol of non-radiolabelled L-methionine. The methionine (final specific radioactivity is 2.77 Ci/mmol) was then dissolved in 45 ml of phosphate-buffered saline supplemented with 0.3% glucose. The incubation was begun by the addition of 45 ml of washed packed erythrocytes suspended in 45 ml of the phosphate-buffered saline containing glucose; the final haemotocrit was 33% and the final L-methionine concentration was 12 μM. The cells were incubated for 3.5 h at 37 °C in a shaking water bath. After this time, cells were pelleted by centrifugation at 5000 *g* for 5 min at 0–4 °C and the supernatant was removed by aspiration. The cell pellet was washed twice more in 10 vol. of phosphate-buffered saline at 0 °C. In order to prevent 'non-physiological' enzymic methylation after cell lysis (Runte *et al.*, 1982), the methyltransferase inhibitor *S*-adenosyl-L-homocysteine (19.2 mg dissolved in 6.5 ml of phosphate-buffered saline) was added to the packed cells. The erythrocytes were then lysed by the addition of 10 vol. of 5 mM-sodium phosphate buffer, pH 6.3, at 0 °C (the *S*-adenosyl-L-homocysteine concentration after lysis was 100 μM). The membranes were removed by centrifugation at 23000 *g* for 10 min at 0–4 °C and the cytosol was retained. The membranes were washed in 50 ml of 5 mM-sodium phosphate buffer, pH 6.3, and the supernatant from the wash was added to the cytosolic fraction. Calmodulin was then purified from the lysate as described above. $CaCl_2$ and 2-mercaptoethanol (final concentrations 5 mM and 1 mM respectively) were added to 75% of the pooled calmodulin-containing eluate from the DEAE-Sephadex step; this material was then subjected to affinity chromatography as described above.

### Methylation of calmodulin in a lysed-cell system *in vitro*

Erythrocytes were methylated in the lysed-cell system *in vitro* described by Terwilliger & Clarke (1981). *S*-Adenosyl-L-[*Me*-$^3$H]methionine (0.067 μmol; 15 Ci/mmol) and 1.42 μmol of non-radiolabelled *S*-adenosyl-L-methionine (the final specific radioactivity was calculated to be 0.67 Ci/mmol) were freeze-dried until just dry in a polyethylene bottle. The incubation was started by the addition of 200 ml of washed packed erythrocytes followed by freeze–thaw lysis of the cells. This lysis was accomplished by plunging the incubation container into a solid-$CO_2$/propan-2-ol bath at approx. −70 °C. The lysed cells were then incubated at 37 °C for 1 h in a shaking water bath. A 450 ml volume of ice-cold 5 mM-sodium phosphate buffer, pH 8, was added after the incubation. The pH of this buffer facilitates the removal of calmodulin and other cytosolic proteins from the lysed-cell membranes (Dodge *et al.*, 1963). After 1 min, 64 ml of 46 mM-sodium citrate buffer, pH 5.43, was added to lower the pH to 6.8 (Terwilliger & Clarke, 1981). The cytosolic pH was adjusted to 6.3 with 1 M-acetic acid in order to minimize hydrolysis of methyl ester linkages. The membranes were then removed by centrifugation and calmodulin was isolated from the resultant cytosol as described above for the experiment studying methylation *in vivo*.

### SDS/polyacrylamide-gel electrophoresis

Two systems were employed.

**System 1.** Electrophoresis was performed in a pH 2.4 buffer system developed by Fairbanks & Avruch (1972) with the modifications described by O'Connor & Clarke (1985). The acidic pH of this gel system prevented the loss of methyl esters from proteins that would be expected to occur at a more basic pH. Samples to be analysed in this system were mixed with an equal volume of sample buffer containing 8% SDS, 0.009% Pyronin Y, 73 mM-2-mercaptoethanol, 24% (v/v) glycerol and 75 mM-sodium phosphate, pH 2.4. The samples were immediately placed in a boiling-water bath for 2 min. The samples were loaded on to 1.4 mm-thick 15 cm × 13 cm polyacrylamide gels (10% acrylamide, 0.345% *NN'*-methylenebisacrylamide); the gels were run at 70 V until the tracking dye reached the lower edge of the gel. The gels were then stained with Coomassie Blue as previously described (Fairbanks *et al.*, 1971).

**System 2.** Electrophoresis was performed with the discontinuous-buffer electrophoretic method of Laemmli (1970). The slightly basic pH of this system would cause hydrolysis of methyl esters, but was useful since EGTA, insoluble in the pH 2.4 gel system, was soluble in this pH range. Samples to be analysed in this system were mixed with an equal volume of sample buffer containing 5% SDS, 146 mM-2-mercaptoethanol, 25% (v/v) glycerol, 0.0025% Bromophenol Blue and 0.13 M-Tris/HCl, pH 6.8. The samples were then loaded on to gels (0.517% *NN'*-methylenebisacrylamide, 15% acrylamide) and run at 30 mA until the tracking dye had passed from the stacking gel into the separating gel. The gels were then run at 60 mA at constant current until the tracking dye reached the lower edge of the gel. The gels were finally stained and destained.

### Protein determination

Protein concentrations were determined by a modified Lowry procedure (Bailey, 1967), with bovine serum albumin as a standard. Protein determinations performed on samples of purified calmodulin were corrected for the difference in relative colour yield between bovine serum albumin and calmodulin; the correction factor used here for calmodulin ($1.163x$, where $x$ is the protein concentration based on bovine serum albumin) was based on amino acid analysis of homogeneous calmodulin (results not shown).

### Radioactivity measurements

Total radioactivity in aqueous samples was determined after the addition of 10–25 vol. of ACS II scintillation cocktail. The number of radiolabelled methyl esters associated with protein was quantified as described by O'Connor & Clarke (1983). In this method, protein-associated methyl esters formed during the incubations performed *in vitro* and *in vivo* were assayed as acid-precipitable base-labile 3-methylbutan-1-ol/toluene-extractable volatile radioactivity. Incorporation of radiolabel into the calmodulin band in the pH 2.4 gel system was detected by slicing the gel lane into 0.4–0.6 mm pieces and placing each piece into a scintillation vial containing 600 μl of NCS tissue solubilizer (Amersham–Searle) and 15 μl of water. The vial was tightly capped

and allowed to incubate at room temperature overnight, with gentle agitation. The vials were then opened, and 9.4 ml of OCS scintillation cocktail (Amersham–Searle) was added, along with 100 $\mu$l of acetic acid. The amount of radioactivity associated with each gel slice was then determined by liquid-scintillation counting.

**Heat treatment and subsequent methylation of calmodulin**

Calmodulin was either partially purified by DEAE-Sephadex chromatography followed by dialysis and freeze-drying (calmodulin dissolved in water to give a final concentration of 1–2 mg of protein/ml; 30–60% pure by SDS/polyacrylamide-gel electrophoresis) or purified to homogeneity by affinity chromatography using Affi-Gel Phenothiazine followed by dialysis and freeze-drying (calmodulin dissolved in water to give a final concentration of 0.2–1.0 mg of protein/ml; > 97% pure by SDS/polyacrylamide-gel electrophoresis). Protein carboxy-group methyltransferase was purified 3000–4000-fold from erythrocyte cytosol by affinity chromatography as described previously (Kim *et al.*, 1978). The enzyme typically displayed a specific activity of 4000 pmol of methyl groups transferred/min per mg with ovalbumin as a substrate at pH 6.0. The protein concentration of the enzyme preparations was in the range 20–30 $\mu$g/ml. The exact conditions under which calmodulin was heated and methylated are described in the appropriate Figure legends. The partially purified calmodulin preparation was used in experiments where the degree of methylation was assessed after SDS/polyacrylamide-gel electrophoresis; we found no significant differences in the methylatability of calmodulin in the two preparations.

**Demethylation of purified [$^3$H]methylated calmodulin**

[$^3$H]Methylated calmodulin was prepared from an aqueous solution of affinity-purified calmodulin that had been heated at 100 °C for 90 min and then methylated as described above. The methylated calmodulin was desalted on a Sephadex G-25 column (1 cm × 48.5 cm) that was equilibrated and subsequently eluted with doubly distilled water. Methylated calmodulin was eluted before and well separated from the *S*-adenosyl-L-[*Me*-$^3$H]methionine. The peak calmodulin-containing fractions from the column were collected, pooled and freeze-dried. Hydrolysis rates were measured by the method of Terwilliger & Clarke (1981) as described in the legend for Fig. 6.

## RESULTS

**Methylation of calmodulin in intact cells**

Calmodulin was purified from intact erythrocytes methylated by incubation with L-[*Me*-$^3$H]methionine as described in the Materials and methods section. When erythrocyte cytosol prepared from these cells was fractionated on a DEAE-Sephadex column, a small peak of radioactivity was found to be co-eluted with the calmodulin activity. The peak calmodulin-containing fractions from the DEAE-Sephadex chromatography were pooled and this material was subjected to affinity chromatography as described in the Materials and methods section. In the presence of $Ca^{2+}$ calmodulin activity was bound to the column but the bulk of the radioactivity was eluted. There was, however, a small but significant peak of radioactivity that was co-eluted with calmodulin activity when the column was eluted with EGTA-containing buffer. Greater than 98% of this radioactivity is associated with protein methyl esters when assayed as described in the Materials and methods section (results not shown).

In order to determine the $M_r$ and purity of the [$^3$H]methylated material that was co-eluted with calmodulin from the affinity column, we fractionated the polypeptides by SDS/polyacrylamide-gel electrophoresis with the pH 2.4 gel system. The results of this experiment, shown in Fig. 1(*a*), reveal a single broad Coomassie Blue-staining band, which runs similarly to authentic calmodulin. All of the radioactivity appears to be associated with this one protein band. In addition, when samples of these same fractions were subjected to SDS/polyacrylamide-gel electrophoresis with the Laemmli (1970) system, all of the Coomassie Blue-staining material was found to exhibit the $Ca^{2+}$-dependent shift in mobility (results not shown) that is characteristic of calmodulin (Burgess *et al.*, 1978).

The number of methyl groups on calmodulin was calculated from the radioactivity associated with this protein fraction. The specific radioactivity of the methyl groups was calculated from the specific radioactivity of the methionine label after a correction was made for dilution by endogenous methionine at 12 $\mu$M (Oden & Clarke, 1983). The number obtained (0.00029 mol of methyl groups/mol of calmodulin) indicates that only a few calmodulin molecules become methylated under these conditions. It is possible, however, that some of the labile methyl esters might be lost during the isolation procedure and that there may not be complete turnover of methyl groups during the 3.5 h incubation period. Both of these effects would lead to an underestimation of the [$^3$H]methylated calmodulin content. From our studies on the hydrolytic lability of methylated calmodulin (see below) and from studies of long-term incubation of erythrocytes with L-[*Me*-$^3$H]methionine (O'Connor & Clarke, 1984), we would not expect either of these effect to be very large. In any case, the value of about three methyl groups per 10000 polypeptide chains should be regarded as a minimum.

**Methylation of calmodulin in the lysed-cell system *in vitro***

Calmodulin was purified from erythrocytes [$^3$H]-methylated in broken cells as described in the Materials and methods section. As in the experiment studying methylation in intact cells, there is a small but significant peak of radioactivity that is co-eluted with calmodulin activity in both the DEAE-Sephadex and the affinity-chromatography steps. Greater than 90% of this radioactivity was found to be associated with protein methyl esters when the fractions were analysed as described in the Materials and methods section.

Fig. 1(*b*) shows the results of subjecting a portion of a peak calmodulin-containing fraction, from the affinity-chromatography stage of the purification, to SDS/polyacrylamide-gel electrophoresis with the pH 2.4 gel system. Radioactivity was found to be associated with the single protein band with $M_r$ approx. 17000. When samples of the peak fractions from the affinity chromatography were subjected to SDS/polyacrylamide-gel electrophoresis as above, all of the protein present exhibited a $Ca^{2+}$-dependent shift in mobility (results not shown). The extent of methylation of calmodulin was

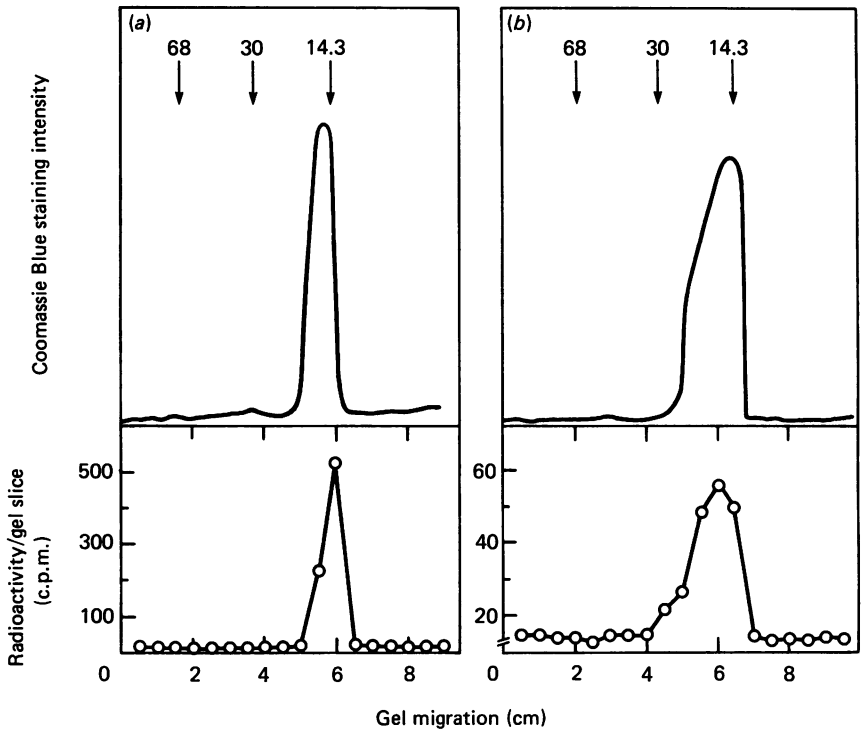


**Fig. 1. Comparison of Coomassie Blue-staining pattern and [$^3$H]methyl-group incorporation of purified erythrocyte calmodulin on pH 2.4 SDS/polyacrylamide-gel electrophoresis**

Samples of affinity-purified [$^3$H]methylated calmodulin from either the methylation reaction with intact cells (*a*) or the methylation reaction *in vitro* (*b*) were dialysed and freeze-dried as described in the Materials and methods section. The samples were then subjected to SDS/polyacrylamide-gel electrophoresis with the pH 2.4 gel system, stained and destained, and densitometric scans were made with a Helena Quick Scan densitometer with a 570 nm filter. The arrows indicate the relative position of $M_r$ standards electrophoresed in parallel lanes; these standards included bovine serum albumin ($M_r$ 68000), carbonic anhydrase ($M_r$ 30000) and lysozyme ($M_r$ 14300). The gel was then sliced into 0.5 cm slices and the radioactivity associated with each gel slice was determined as described in the Materials and methods section.

determined as described for the sample from the incubation with intact cells. After correction for dilution of the *S*-adenosyl-L-[*Me*-$^3$H]methionine pool by endogenous *S*-adenosyl-L-methionine (3.5 $\mu$M; Oden & Clarke, 1983), we calculated that there is at least 0.00028 mol of methyl groups/mol of calmodulin. This value is similar to that obtained for calmodulin in intact cells.

### Calmodulin methylatability and activity as a function of time of heating at 100 °C

Assuming that the limited ability of calmodulin to serve as a methyl-group acceptor was due to the small number of molecules containing D-aspartate and L-isoaspartate residues formed by spontaneous degradation reactions, we asked if the accelerated degradation brought on by heating calmodulin might result in an increased number of methyl-group-accepting sites. In the experiment shown in Fig. 2 we found that the methylatability of calmodulin increases as a function of the time of heating at 100 °C before methylation; the maximal incorporation was approx. 0.0095 mol of methyl groups/mol of calmodulin at 80 min at pH values of 5 and 7 (Fig. 2*a*).

It was possible that the heat-dependent increase in calmodulin methylation seen in Fig. 2 might be due in part to a loss of endogenous methyl esters, which would open new sites for methylation reactions. To test this, we performed an experiment similar to the previous one, heating calmodulin for 15 min at either 37 °C or 100 °C in the presence of buffers of various pH values. Hydrolysis kinetics determined for methylated calmodulin incubated at 37 °C, described below, indicate that less than 10% of the methyl groups would be removed by preincubation at pH 7.5 for 15 min, whereas 50% would be removed by incubation at pH 9.0 for 15 min. The fact that there was no increase in the methyl-group-acceptor activity of calmodulin pretreated at pH values from 5 to 10 (Fig. 3) indicates that methyl-group incorporation is not limited by the presence of endogenous methyl esters. The increase in the methyl-group-acceptor ability of calmodulin heated at 100 °C, under conditions where the reaction is only limited by the concentration of the protein substrate, was found to be essentially independent of the pH over the range 5–9 (Fig. 3).

We were also interested in exploring whether the presence of $Ca^{2+}$ might affect the increase in methyl-group-acceptor activity in heated calmodulin. Calmodulin is more resistant to proteolysis in the presence of $Ca^{2+}$, and undergoes a substantial change in conformation upon binding of $Ca^{2+}$ (Klee & Vanaman, 1982). In addition, Runte *et al.* (1982) have reported that the extent of methyl-group incorporation into calmodulin *in vitro* is dependent upon the concentration of various cations during methylation. We have determined the effects of changing the free $Ca^{2+}$ concentration, during a preincubation at 100 °C, upon the heat-induced increase in

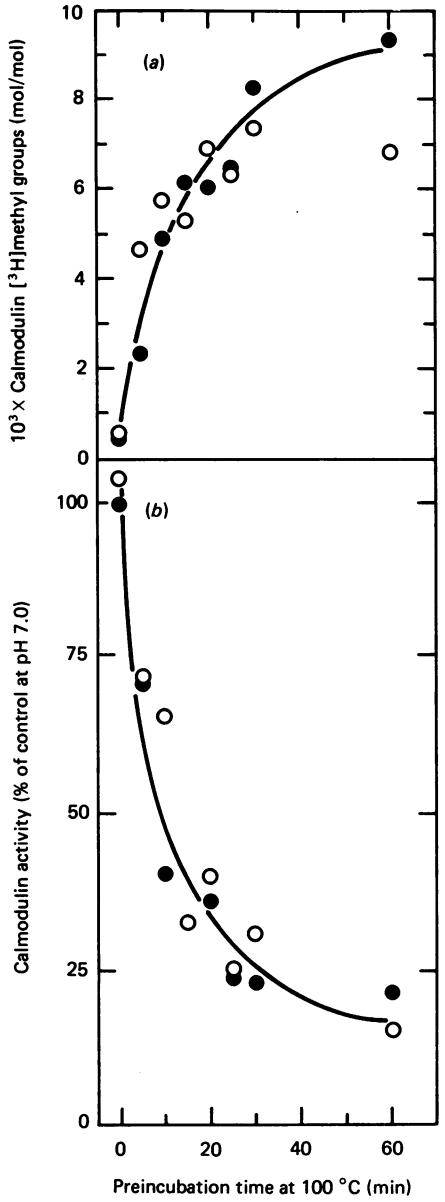


**Fig. 2. Change in calmodulin methylatability as a function of time of heating at 100 °C at pH 5.0 and 7.0**

Dialysed and freeze-dried calmodulin, purified by DEAE-Sephadex chromatography (28 $\mu$g of protein, 57% pure by SDS/polyacrylamide-gel electrophoresis), was dissolved in distilled water and heated at 100 °C in sealed polypropylene tubes containing 200 mM-ammonium acetate at either pH 5.0 (○) or pH 7.0 (●). Methylation was quantified by incubating freeze-dried samples of heated calmodulin with partially purified erythrocyte protein methyltransferase, *S*-adenosyl-L-[$^3$H]methionine (5 Ci/mmol; 11.3 $\mu$M final concentration in incubation mixture) and 40 mM-sodium acetate buffer, pH 6.0, for 100 min at 37 °C. After the incubation, *S*-adenosyl-L-homocysteine (70 $\mu$M final concentration) was added to stop the reaction, and a portion of each sample was assayed for calmodulin activity; the remainder of the sample was subjected to pH 2.4 SDS/polyacrylamide-gel electrophoresis. The radioactivity associated with the calmodulin region of each gel lane was then determined as described in the Materials and methods section. Data are presented in terms of mol of [$^3$H]methyl groups incorporated/mol of calmodulin.

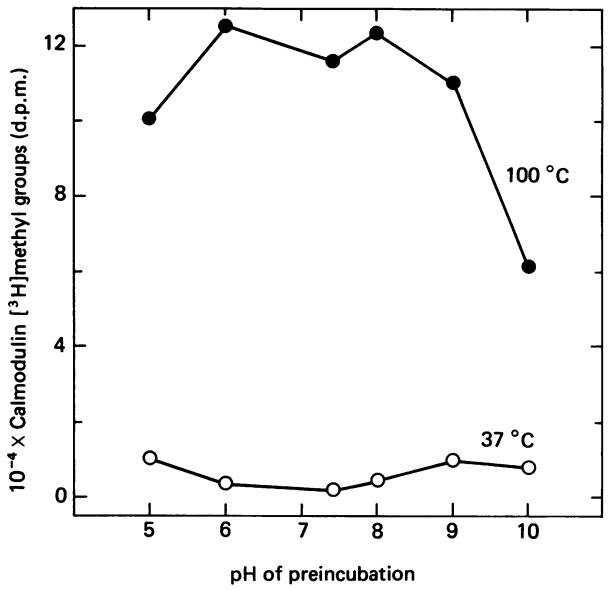


**Fig. 3. Change in calmodulin methylatability as a function of pH of preincubation at 37 °C or 100 °C**

Samples of calmodulin [44 $\mu$g of protein, partially purified by DEAE-Sephadex chromatography (45% pure) and dissolved in distilled water after dialysis and freeze-drying] were heated for 15 min at 100 °C in sealed polypropylene tubes containing 11 mM-ammonium acetate buffer, pH 5.0 or 6.0, or 11 mM-ammonium bicarbonate buffer, pH 7.4, 8.0, 9.0 or 10.0. Control samples, incubated for 15 min at 37 °C, were also prepared. The buffer salts were then removed by freeze-drying and the samples were methylated as described in Fig. 2 legend. The samples were then subjected to pH 2.4 SDS/polyacrylamide-gel electrophoresis, and the radioactivity associated with the calmodulin-containing portion of the gel lanes was determined as described in the Materials and methods section. ●, Samples preincubated at 100 °C; ○, samples preincubated at 37 °C.

calmodulin methylatability. In an experiment similar to that shown in Fig. 2, we extended the time course of the 100 °C heat treatment of calmodulin in either 0.9 mM-$Ca^{2+}$ or 0.9 mM-EGTA (Fig. 4). We found no apparent difference in the decrease in calmodulin activity between samples heated in the presence and in the absence of $Ca^{2+}$. The methylatability of calmodulin was found to increase as a function of time of heating, finally levelling off at approx. 2.5 h (maximum incorporation approx. 0.08 mol of methyl groups/mol of calmodulin); the greatest increases in methylatability occur during the first hour of heating, while calmodulin still retains a fraction of its biological activity. The presence or absence of $Ca^{2+}$ during the heating appears to have little or no effect on this phenomenon.

### Determination of the rate of protein aspartate/asparagine residue racemization in calmodulin and ribonuclease A

In order to determine the correlation between the time-dependent increase in calmodulin methylatability upon heating and its content of residues that give D-aspartate upon hydrolysis, we heated calmodulin at 100 °C for various lengths of time in 200 mM-ammonium acetate buffer, pH 6.0. The D-/L-aspartate ratio was determined for acid hydrolysates. From the data in Fig. 5,

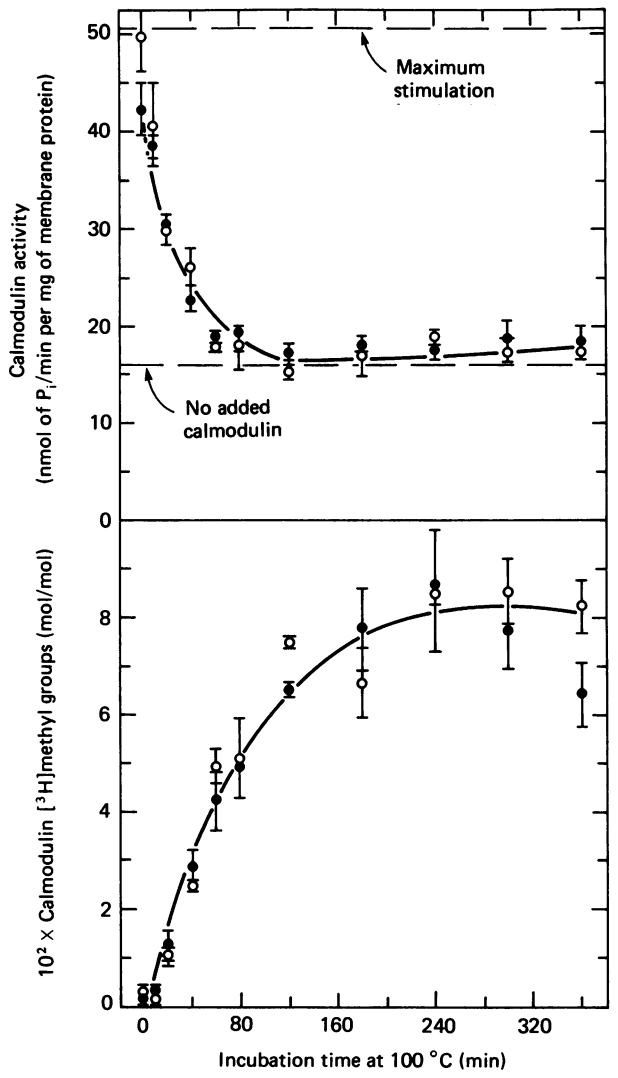


**Fig. 4. Determination of the change in calmodulin methylatability and activity as a function of heating time in the presence of $Ca^{2+}$ or EGTA**

Samples (2.3 $\mu$g) of homogeneous calmodulin were sealed in polypropylene tubes containing 0.9 mM-EGTA (●) or $CaCl_2$ (○) and 142 mM-Mes buffer, pH 6.0; the tubes were then heated at 100 °C in a water bath for various lengths of time. After the tubes had been cooled on ice, $CaCl_2$ (final concentration of added $Ca^{2+}$ 0.9 mM) was added to the tubes that had been heated in the presence of EGTA; EGTA (final concentration of added EGTA 0.9 mM) was added to the tubes that had been heated in the presence of $CaCl_2$ in order to give the same free $Ca^{2+}$ concentration in all samples before methylation and later calmodulin assays. Portions of each sample were assayed for calmodulin activity. The remainder of the sample was methylated by incubation for 100 min at 37 °C after the addition of partially purified erythrocyte protein carboxy-group methyltransferase dissolved in 5 mM-EDTA/5 mM-sodium phosphate buffer, pH 6.7 (final EDTA concentration is 0.37 mM) and *S*-adenosyl-L-[$^3$H]methionine (5 Ci/mmol; 5 $\mu$M final concentration in incubation mixture). The incorporation of $^3$H radioactivity into protein methyl esters, expressed as mol of methyl groups/mol of calmodulin, was determined as described in the Materials and methods section.

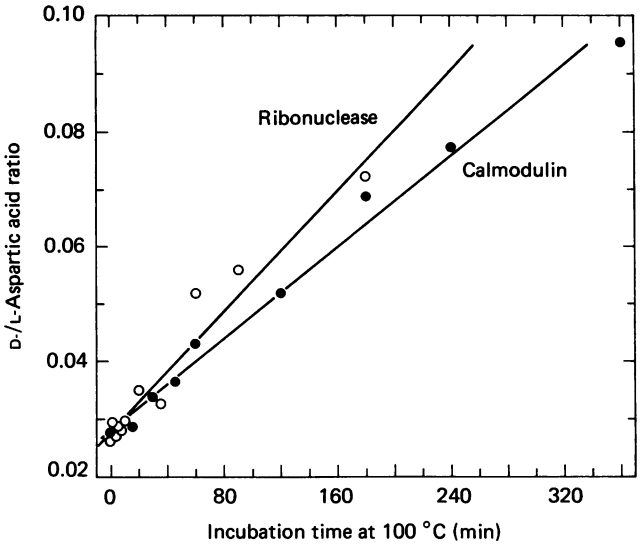


**Fig. 5. Kinetics of accumulation of D-aspartate/D-asparagine residues in calmodulin and ribonuclease as a function of time of heating at 100 °C**

Samples (49 $\mu$g) of homogeneous calmodulin (●), dissolved in distilled water after dialysis and freeze-drying, were sealed in glass hydrolysis tubes containing 200 mM-ammonium acetate buffer, pH 6.0. Samples (1 mg) of ribonuclease A (○), dissolved in 0.1 M-sodium phosphate buffer, pH 5.36, were also sealed in glass hydrolysis tubes. The samples were then heated at 100 °C in a water bath for the indicated time. The tubes were then opened and the contents subjected to acid hydrolysis in 6 M-HCl for 6 h at 108 °C *in vacuo*. Aspartate was then purified from the hydrolysate by ion-exchange chromatography, and the D-/L-aspartate ratio was determined by the method of Aswad (1984*a*) as described by Murray & Clarke (1984).

we detect a time-dependent increase in the D-/L-aspartate ratio of calmodulin. Using the method of Friedman & Masters (1982), we calculated that the racemization rate constant, $k_{rac.}$, for calmodulin at 100 °C is $3.29 \times 10^{-6}$/s. It had been found that ribonuclease A exhibits a similar increase in methylatability when heated for short periods of time (S. Clarke, unpublished work). We therefore carried out parallel experiments using ribonuclease A, which was heated at 100 °C in 0.2 M-sodium phosphate buffer, pH 5.36. The corresponding value for the $k_{rac.}$ of ribonuclease A is $4.47 \times 10^{-6}$/s. In both cases there is a parallel increase in methyl-group-acceptor ability and content of D-aspartate/D-asparagine/D-isoaspartate residues.

### Site of methylation of calmodulin: determination of rates of hydrolysis of [$^3$H]methylated calmodulin

When purified [$^3$H]methylated calmodulin was digested with carboxypeptidase Y under conditions that lead to the release of D-aspartic acid $\beta$-[$^3$H]methyl ester for erythrocyte membrane and cytosolic proteins (Clarke *et al.*, 1984), we were unable to detect any radiolabelled aspartic acid $\beta$-methyl ester or glutamic acid $\gamma$-methyl ester. We have also been unsuccessful so far in isolating free or peptide-bound aspartic acid $\alpha$-methyl esters from such digestions. Therefore, to obtain information on the possible chemical nature of the methyl esters in calmodulin, we determined the rate of demethylation of

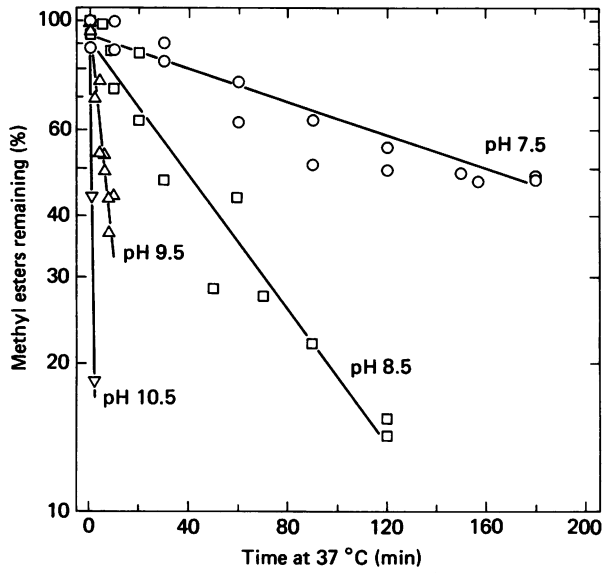


**Fig. 6. Determination of hydrolysis rates of [$^3$H]methylated calmodulin**

Desalted [$^3$H]methylated calmodulin was prepared and freeze-dried as described in the Materials and methods section. The freeze-dried material was dissolved in a minimum amount of water (211 $\mu$g/ml; 356 c.p.m./$\mu$l); 100 $\mu$l samples were placed in capped polypropylene tubes. At zero time the samples were diluted with 1 ml of one of the following buffers (pH measured at 23 °C): 0.1 M-sodium phosphate buffer, pH 7.5 (○); 0.1 M-Tris/HCl buffer, pH 8.5 (□); 0.1 M-glycine/NaOH buffer, pH 9.5 (△); 0.1 M-glycine/NaOH buffer, pH 10.5 (▽). At various times 50 $\mu$l samples were withdrawn from each incubation mixture and the reaction was stopped by the addition of 75 $\mu$l of 2 M-HCl at 0–4 °C. Samples were then freeze-dried to remove the [$^3$H]methanol formed during hydrolysis. The non-volatile material remaining was then taken up in 100 $\mu$l of water; 2.5 ml of ACS II was then added and the radioactivity of the sample was counted by liquid-scintillation spectrophotometry. Lines were drawn from least-squares fits of the data points.

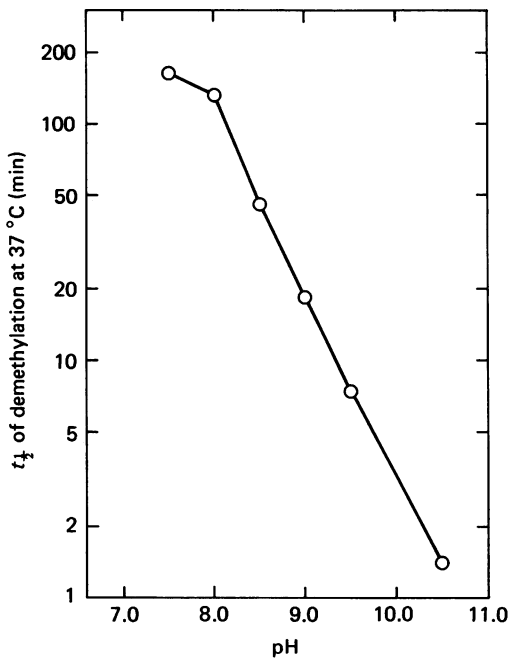


**Fig. 7. Half-times of spontaneous demethylation of enzymically methylated erythrocyte calmodulin as a function of pH at 37 °C**

Values were obtained from least-squares fits of data at pH 7.5, 8.5, 9.5 and 10.5 from Fig. 6. Additional values at pH 8.0 and 9.0 were determined from similar data obtained with 0.1 M-Tris/HCl buffer and 0.1 M-glycine/NaOH buffer respectively.

[$^3$H]methylated calmodulin at various pH values (Figs. 6 and 7). Although it is not possible to distinguish isoaspartic acid $\alpha$-methyl ester from aspartic acid $\beta$-methyl ester by this procedure, we did find that the demethylation data could be adequately explained in terms of one kinetic class of sites; there was also a linear dependence on the rate of demethylation and the pH. These features are not found when demethylation of erythrocyte membrane proteins is measured (Terwilliger & Clarke, 1981; Barber & Clarke, 1985), and this indicates that these membranes contain a more heterogeneous collection of methyl ester groupings.

## DISCUSSION

We have measured the extent of the methylation of carboxy groups of calmodulin in intact human erythrocytes. We found that the incorporation is very small, representing only about 0.00029 mol of methyl groups/mol of calmodulin polypeptide after a 3.5 h incubation. This value is comparable with values of 0.00010–0.00032 mol/mol that can be estimated from the data of Runte *et al.* (1982). [This estimate is based on the assumptions that the concentration of calmodulin in erythrocytes is 2.5 $\mu$M (Sobue *et al.*, 1981), that the label, [*Me*-$^3$H]methionine, is not diluted by endogenous methionine, and that the recovery of calmodulin after DEAE-cellulose chromatography and fluphenazine–Sepharose 4B affinity chromatography is similar to that in our purification system (10–33%).] We have also shown that erythrocyte calmodulin can be methylated *in vitro* to a similar degree. The extent of these methylation reactions are, however, much smaller than those reported with non-erythrocyte calmodulin; the range of previous values is 0.01 mol of methyl groups/mol of calmodulin polypeptide chain (Johnson *et al.*, 1985) to 0.50 mol of methyl groups/mol of polypeptide chain (Gagnon *et al.*, 1981). It may be noted that the purification of calmodulin in some of the previous studies involved a brief 80–100 °C heat treatment or exposure to buffers of mild alkaline pH (Runte *et al.*, 1982; Billingsley *et al.*, 1983, 1984). Since we detect a marked increase in the methyl-group-acceptor activity of erythrocyte calmodulin with heating at 100 °C, and others have detected increases on treatment with base at 37 °C (Johnson *et al.*, 1985) and 50 °C (Murtaugh *et al.*, 1983), it is possible that the methyl-group-acceptor ability of calmodulin in these previous preparations had been increased, in comparison with 'native' calmodulin, as an artifact of isolation.

One of the major functions of calmodulin in the erythrocyte is the activation of the membrane ($Ca^{2+}+Mg^{2+}$)-stimulated ATPase (Gopinath & Vincenzi, 1977). One can calculate from the data presented here that only 46

methylated calmodulin molecules would be present in an erythrocyte, which contains about 160000 calmodulin molecules (Sobue *et al.*, 1981) and 400 ($Ca^{2+}+Mg^{2+}$)-stimulated ATPase molecules (Drickamer, 1975). Even if the methylation of calmodulin resulted in changes in the affinity of calmodulin for the ATPase or a diminished ability to activate the enzyme, it is hard to imagine that the carboxy-group methylation of only a few calmodulin molecules could exert a physiological change *in vivo*. It is possible that the measured extent of methyl-group incorporation represents a basal level (i.e. methylation of calmodulin may be increased by some physiological stimulus absent under our incubation conditions), but no such stimulus has been detected to date. We have performed preliminary experiments that indicate that methylation of erythrocyte calmodulin *in vitro* with *S*-adenosyl-L-methionine and partially purified methyltransferase does not in fact significantly alter its ability to activate the erythrocyte ($Ca^{2+}+Mg^{2+}$)-stimulated ATPase (results not shown). Thus the physiological significance of calmodulin carboxy-group methylation is not clear at the present time. It should be noted that calmodulin is not one of the major methyl-group acceptors in intact erythrocytes, either in terms of the total number of methyl esters or in the stoichiometry of methylation (Table 1).

It has been suggested that methylation of erythrocyte proteins occurs at aspartate residues present in the uncommon D configuration or as L-isoaspartate residues (for a review see Clarke, 1985). These residues are believed to arise from spontaneous cellular racemization and/or isomerization reactions, which may proceed via succinimide intermediates (McFadden & Clarke, 1982; Aswad, 1984*b*; Murray & Clarke, 1984). If calmodulin is, indeed, methylated at D-aspartate and L-isoaspartate residues, then increasing its content of such groups should result in an increase in calmodulin's ability to act as a methyl-group acceptor. On the basis of the initial rate of D-aspartate/D-asparagine/D-isoaspartate accumulation (Fig. 5) and of the initial rate of the increased methyl-group-acceptor ability (Fig. 2) of calmodulin heated at 100 °C, we can calculate that only a maximum of 10% of these racemized residues can be methylated. This comparison is complicated because one might not expect that the enzyme would recognize both D-aspartate and D-asparagine (or D-isoaspartate) residues, and because some (or all) of the methylation sites may be at L-isoaspartate residues. Nevertheless, this result suggests that not all susceptible residues may be accessible to the enzyme. The observation that the rate of increase in calmodulin methylatability during heating decreases once all of the calmodulin activity has been lost (Fig. 4) suggests that the ability of calmodulin to serve as a methyl-group acceptor may be a function of both its content of racemized/isomerized aspartate/asparagine residues as well as its conformation.

Recent work has shown that mild treatment with base, which does not cause significant racemization of adrenocorticotropic hormone, greatly enhances the methyl-group-acceptor ability of bovine brain calmodulin (Johnson *et al.*, 1985). Furthermore, chemical modification of the asparagine and glutamine residues was found to diminish significantly this enhancement of calmodulin methylation under basic conditions, suggesting that the major sites of methylation in brain calmodulin were at L-isoaspartate residues resulting from the deamidation of L-asparagine residues. Although much of the evidence from our studies with erythrocyte calmodulin is consistent with this picture, our finding that the increase in methylatability with heating is independent of pH in the range 5–9 (Fig. 3) might not be expected if the major methylation sites were derived from asparagine residues, because these residues might be expected to accumulate succinimides (and isoaspartate residues) more rapidly at basic pH. On the other hand, since succinimide formation from aspartate residues would appear to require the protonated form of the carboxy group, any increase in the nucleophilicity of the peptide nitrogen atom due to base would be equally offset by the loss of this free carboxylic acid. These considerations would suggest that the methylation sites of erythrocyte calmodulin were derived from aspartate and not asparagine residues. It is possible that treatment with base or heating at neutral pH generates different methylation sites.

Measurement of the spontaneous demethylation rate of erythrocyte calmodulin (Figs. 6 and 7) indicates that there is only one kinetic class of methyl-group-acceptor sites. The rate of demethylation at pH 7.4 ($t_{\frac{1}{2}}$ = 170 min) is intermediate between those of the erythrocyte membrane and cytosolic proteins (O'Connor & Clarke, 1984) and is significantly lower than that measured for two related peptides containing $\alpha$-methyl esters at L-isoaspartyl-glycyl sequences ($t_{\frac{1}{2}}$ = 4–7.2 min; Johnson & Aswad, 1985; Murray & Clarke, 1986). Although this comparison might suggest that the erythrocyte calmodulin methylation site may not be at an Asp-Gly sequence, the effects of other adjacent amino acid residues and of the conformation of the peptide on the demethylation rate have not yet been established.

Because the formation of protein succinimides from aspartate and asparagine residues may represent intermediates on the route to both D-aspartate and L-isoaspartate residues (Clarke, 1985), it would be very useful to be able to predict from both the sequence and the three-dimensional structure which residues would be likely to form succinimides. Two criteria can be used to identify such residues. In the first place, it appears that a glycine or similar small residue on the carboxy side of the aspartate or asaparagine residue may enhance imide formation. Evidence for this comes from studies of imide

**Table 1. Protein methyl esters in intact human erythrocytes**

| Protein | $10^3 \times$ Degree of methylation (mol of methyl groups/mol of polypeptide) | Methyl ester groups per cell |
|---|---|---|
| Calmodulin* | 0.29 | 46 |
| Carbonic anhydrase† | 0.061 | 1400 |
| Haemoglobin† | 0.003 | 3400 |
| Band 2.1 protein‡ | 78 | 7800 |
| Band 3 protein‡ | 5.8 | 5800 |
| Band 4.1 protein‡ | 16 | 3200 |

* Present study.
† O'Connor & Clarke (1984).
‡ Data on degree of methylation from Barber & Clarke (1983); methyl ester groups/cell calculated by using values for the number of these proteins in erythrocytes reported by Bennett (1985).

formation from peptide aspartic acid $\beta$-benzyl esters (Bodanszky & Kwei, 1978; Capasso *et al.*, 1984) and studies on the hydroxylamine-mediated cleavage of Asn-Gly sequences in proteins (Bornstein, 1970; Blodgett *et al.*, 1985). A second criteria of imide formation is the relative position of the peptide nitrogen atom and the side-chain carbonyl carbon of the aspartate or asparagine residues. Succinimide formation is favoured when these atoms are in proximity, which is maximized when the dihedral angle $\psi$ is $-120°$ and the dihedral angle $\chi_1$ is 120°. Recently, a structure of rat testes calmodulin was obtained at a resolution of 0.3 nm (3.0 Å) (Babu *et al.*, 1985). Although the side-chain conformational angles $\chi_1$ were not determined, $\psi$ values were obtained for the aspartate and asparagine residues (Y. S. Babu, C. E. Bugg & W. J. Cook, personal communication). By this criterion alone, two Asp-Lys sequences (20–21 and 93–94) and one Asn-Tyr sequence (137–138) might be sites of succinimide formation in the native structure because their $\psi$ angles are within 13° of $-120°$. On the other hand, one Asp-Gly sequence (58–59) has a $\psi$ value that deviates from $-120°$ by 36°; the other Asp-Gly sequences and the Asn-Gly sequences vary from it by 101–171°, and these latter sequences might not contribute to succinimide formation. Knowledge of the actual sites of L-isoaspartate and/or D-aspartate residue formation in calmodulin will allow us to understand the relative importance of the polypeptide conformation and the amino acid sequence for succinimide formation at aspartate and asparagine residues.

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