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Carboxylmethylation affects the proteolysis of myelin basic protein by *Staphylococcus aureus* V_8 proteinase

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Bovine myelin basic protein (MBP), charge isoform 1 (C1) was carboxylmethylated by the enzyme D-aspartyl/L-isoaspartyl protein methyltransferase (EC. 2.1.1.77) and the carboxylmethylated protein was subjected to proteolysis by sequencing grade staphylococcal V_8 proteinase at pH 4.0 to identify its carboxylmethylated modified aspartate and/or asparagine residues which are recognized by this methyltransferase. Native MBP, C1 was treated similarly and the proteolysis products were compared, using electrophoretic, chromatographic and amino acid sequencing techniques. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) revealed differences in the kinetics of proteolysis between the native and the carboxylmethylated MBP, C1 which were confirmed using HPLC. Partial sequencing of the native and carboxylmethylated fragments eluting at about 29 min (P_{29}) revealed cleavage of native MBP, C1 at Gly-127–Gly-128 and of the carboxylmethylated MBP, C1 at Phe-124–Gly-125. Additional evidence including tryptic subdigestion of carboxylmethylated P_{29} disclosed the following partial sequence for this peptide: Gly-Tyr-Gly-Gly-Arg-Ala-Ser-Asp-Tyr-Lys-Ser-Ala-His-Lys-Gly-Leu-Lys-Gly-His-Asp-Ala-Gln-Gly-Thr-Leu-Ser-Lys-Ileu-Phe-Lys-. This sequence matches MBP residues 125–154. As a result of these findings, Asp-132 and Asp-144 were identified as two of the modified (isomerized or racemized) methyl-accepting L-aspartates in MBP. The results of the proteolysis experiments wherein the sequencing grade staphylococcal V_8 proteinase was used at the rarely tested pH of 4.0, rather than at its commonly tested pH of 7.8, also disclose that the proteinase totally failed to recognize and hence cleave the two Glu-X bonds (Glu-82–Asn-83 and Glu-118–Gly-119) of MBP, preferring to cleave the protein at a number of hitherto unreported sites.

Introduction

The carboxylmethylation of eukaryotic proteins is catalyzed by protein D-aspartyl/L-isoaspartyl methyltransferase (EC. 2.1.1.77, *S*-adenosyl-L-methionine: protein-D-aspartate *O*-methyl-transferase) (PM II) [1–3]. PM II recognizes as carboxylmethylation sites in proteins two atypical forms of L-aspartate, L-iso-aspartate and D-aspartate [4,5] which appear to exist in proteins at low levels and, in mouse brain, as early as 19 days gestationally [6]. A detailed analysis of the origin of L-iso-aspartate in proteins has revealed L-aspartate and L-asparagine as its potential sources. Work on two purified proteins, glucagon [7] and calmodulin [8], has disclosed the highly selective nature of the L-iso-aspartate generating process, inasmuch as only 1 of the 3 (glucagon) and 2 of the 16 (calmodulin) available L-aspartates furnish L-iso-aspartate. Similarly, the single L-asparagine of glucagon [7] but none of the 6 L-asparagines of calmodulin [8] exist as L-iso-aspartate in the native proteins. With regard to D-aspartate, Shapira et al. [9] have shown every L-aspartate and L-asparagine of human myelin basic protein (MBP) to exist as D-aspartate, ranging in amount from a low of 2.2% for L-asparagine-92 to a high of 17.7% for L-aspartate-160.

Carboxylmethylation sites in proteins may also be generated by a number of in vitro treatments which bring about the conversion of susceptible L-aspartates and L-asparagines to L-iso-aspartate. For example, alkaline treatment of glucagon and calmodulin converted L-asparagine-60 and/or L-asparagine-97 of calmodulin

Abbreviations: MBP, myelin basic protein; C1, charge isoform 1; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PM II, protein D-aspartyl/L-isoaspartyl methyltransferase; SAM, *S*-adenosyl-L-methionine; NS, non-sequencing; S, sequencing.

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[10] and L-asparagine-28 of glucagon [8] into L-iso-aspartate, while heating calmodulin [11] generated L-iso-aspartate from L-aspartate, both treatments resulting in significant increases in the proteins' carboxylmethylation.

MBP, the most abundant protein of brain white matter [12,13], is an excellent carboxylmethyl-accepting substrate for PM II [14]. The high efficiency of its carboxyl-^{3}H-methylation was recently confirmed in our laboratory [15].

In this paper we define a number of additional parameters which influence the carboxyl-^{3}H-methylation of MBP in vitro. Additionally, we document differences between native and carboxylmethylated MBP as substrates for *Staphylococcus aureus* V_8 proteinase, we identify modified (isomerized or racemized) Asp-132 and Asp-144 as two of the methyl-accepting residues of MBP and we describe an atypical mode of proteolysis of this protein by the *S. aureus* V_8 proteinase at the rarely tested pH of 4.0.

Materials and Methods

Materials

S-Adenosyl-L-methionine (di-*p*-toluenesulfonate salt) was a gift from Dr. G. Stramentinoli, BioResearch, Liscate, Italy. Crystalline ovalbumin, bovine serum albumin, Coomassie Blue G-250, trypsin (bovine pancreas), treated with diphenylcarbamyl chloride, soybean trypsin inhibitor, α-lactalbumin, β-lactoglobulin, aprotinin (bovine lung) and phenyl-methylsulfonyl fluoride were from Sigma (St. Louis, MO) or Pharmacia-LKB (Bromma, Sweden). *S*-Adenosyl-L[*methyl*-^{3}H] methionine (SAM) (1 mCi/ml) with a nominal specific radioactivity of 10–15 Ci/mmol was from ICN Radiochemicals, Irvine, CA or New England Nuclear Research Products, Boston, MA. Several sources and, for a given source, more than one grade of the *Staphylococcus aureus* V_8 proteinase were used. While Miles Scientific, *S. aureus* V_8 proteinase (code No. 39-900-1), the product referred to by Chan et al. [16] was unavailable, Sigma (St. Louis, MO) product No. P8400 (Type XVII) or product No. 791156 from Boehringer (Indianapolis, IN) were used initially and are denoted as non-sequencing (NS) grade V_8 in the text. These were replaced, for most of the sequencing work described in this paper, by sequencing (S) grade *S. aureus* V_8 proteinase, Boehringer product No. 1047817. It should be noted that the specification sheet accompanying this product provides proof of the purity of the product, for 20 μg of the protein elute at A_{215} nm as the only peak of absorbing material when run by reverse phase HPLC under conditions where less than 1% impurity would have been readily detected.

All reagents for electrophoresis were from Hoefer Scientific Instruments, San Francisco, CA. All other reagents were of analytical or HPLC grade. The bovine brain MBP charge isoform C1 (MBP, C1) [17] was provided by Drs. R. Zand and C. Caamaño (Biophysics Division, Institute of Science and Technology, University of Michigan, Ann Arbor, MI).

Peptide '1856': H-Gly-Ser-Leu-Pro-Gln-Lys-Ser-Gln-Arg-Ser-Gln-Asp-Glu-Asn-Gly-OH was a gift from Dr. G. Hashim, St. Luke's Hospital, New York City, NY. Human brain MBP was purified by the procedure of Bellini et al. [18].

Methods

Purification of protein methyltransferase II

PM II was purified from bovine brain cytosol by the procedure of Billingsley and Roth [19]. The final preparation was stored at −70 °C in 50 μl aliquots/tube. Volumes of 1–5 μl/assay were used; PM II remained fully active over a 2-year period.

The heat treatment of MBP, C1

Generally, 500 μl of MBP, C1 (0.8–1 mg/ml) were heated in triple-distilled water (pH 5–6) at 100 °C for periods of time varying between 10 and 45 min. We determined, in the course of this study, that up to a 4-fold increase in carboxylmethylation resulted from a 10 min heat treatment. After lyophilizing the tube contents, the dry residue was suspended in triple distilled water for further use.

The alkaline treatment of MBP, C1

Enough 0.55 M ammonia was added to aqueous solutions of MBP, C1 (0.8–1 mg/ml) to reach pH 11.0. After incubation at 37 °C for 3 h, the pH was lowered to 5.0 with 1 M acetic acid and the tube contents were lyophilized. The dry residue was suspended in triple distilled water for further use.

The carboxylmethylation assay

The procedure of Diliberto and Axelrod [20], as outlined in detail previously [21,22] was used for the quantitative determination of the transfer of ^{3}H-methyl groups from [^{3}H]SAM to MBP, C1. The final assay volume ranged between 30 and 90 μl. Unless otherwise noted, the concentrations of MBP and SAM were 10 μM and 20 μM, respectively. Incubations were at 37 °C for up to 60 min. PM II was present at 11.8 μM (M_r 25000). For other details, refer to the legend to Fig. 1.

The separation of the carboxyl ^{3}H-methylated MBP, C1 from the assay reactants

To separate the radiolabelled MBP, C1 from PM II and [*methyl*-^{3}H]-SAM, carboxylmethylation incubates were diluted to about 175 μl with 0.05% trifluoroacetic acid in water and the radiolabelled MBP, C1 was

separated from the reactants by the HPLC procedure described by Chan et al. [16].

HPLC of MBP, C1

HPLC (LKB Produkter, Bromma, Sweden) of native and carboxyl-^{3}H-methylated MBP, C1 was performed on a Vydac C_{18} reverse phase column (0.4 × 25 cm) (Vydac Corp., Hesperia, CA) [16], except that the concentration of acetonitrile in buffer B was lowered from 60 to 40% (v/v). Absorbance of the effluent was monitored at 226 nm. The flow rate was 1 ml/min and 0.5 ml fractions were collected. Their radioactivity was determined after addition of 4 ml of scintillation cocktail.

Proteolytic fragmentation of MBP, C1

(A) With S. aureus V_8 proteinase. Native and carboxyl-^{3}H-methylated MBP, C1 were digested in a total volume of less than 70 μl in the presence of 5 μg of non-sequencing (NS) or 1 μg of sequencing (S) grade *S. aureus* V_8 proteinase (see Materials and Results) in freshly prepared 40 mM ammonim acetate (pH 4.0). At the end of incubation, the digest was placed on a Vydac C_{18} reverse phase column and the peptides separated using a gradient obtained by mixing 0.05% trifluoroacetic acid (TFA) in water (A) with 0.05% TFA in 40% (v/v) acetonitrile (B) over a 45 min period at a flow rate of 1 ml/min. We verified separately that, at pH 4.0 the sequencing grade V_8 proteinase cleaved insulin B_{ox} (The Boehringer Co. test substrate) to produce the same three peptides it generates at pH 7.8, namely Phe-Val-Asn-Gln-His-Leu-Cys-Gly-Ser-His-Leu-Val-Glu (1–13), Ala-Leu-Tyr-Leu-Val-Cys-Glys-Glu (14–21) and Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Ala (22–30).

(B) With trypsin. Typically, 5–40 μg of carboxyl-^{3}H-methylated MBP, C1 + 30–120 μg of native MBP, C1 were mixed in 0.2 M sodium citrate (pH 6.0) and were digested in the presence of 2.4 μg of trypsin put up in 1 mM HCl [8] for 40 min at 37 °C. The digest was placed on the Vydac C_{18} column and the peptides were separated using the protocol described by Shapira et al. [9]. The presence of carboxyl-^{3}H-methylated peptides was determined by direct counting and, in some instances, by performing the carboxylmethylation assay [21] on the radiolabelled tubes, to verify the alkaline-labile nature of the radioactive product contained in them. The tryptic subdigestion of the (S) V_8 proteinase peptide P_{29} was carried out similarly, in the presence of 2 μg of trypsin at pH 6.0 and in a volume of 40 μl for 40 min at 37 °C. For additional details, see the legend to Fig. 6.

Electrophoresis

(A) SDS-PAGE. This was carried out according to Chantry and Glynn [23] on 1.5 mm slabs in the Hoefer CE 400 vertical slab gel unit. Amounts of protein ranging from 5–80 μg/well were electrophoresed at room temperature. The gels were stained in 0.25% Coomassie Blue G-250 in 10% acetic acid/50% methanol for up to 4 h. Destaining was in 10% acetic acid/50% methanol.

(B) Acetic acid-urea PAGE. This was performed according to Chantry and Glynn [23] except that the concentration of the running and stacking gels was reduced to 7.5% and 3.75%, respectively, for the 14 × 16 cm × 1.5 mm slabs. These gels were run at 200 V (50–60 mA) for 4 h. Staining was for overnight and destaining for about 7 h.

(C) Alkaline urea gels. Disc gels (3 mm I.D.) were prepared according to Cheifetz and Moscarello [24] and were pre-electrophoresed, before loading the samples, for 1.5 h at 3.75 mA/gel. After loading the samples (30–60 μg/tube) electrophoresis was for 3.5 h, also at 3.75 mA/gel. Staining was for 3.5 h as above in 'A' and destaining was for 12 h.

Protein determination

The procedure of Peterson [25] was used, with crystalline bovine serum albumin as standard.

Amino acid sequencing

The University of Michigan Medical School Protein Sequencing Facility performed the sequencing. A minimum of three and a maximum of six cycles (Edman) yielded the relevant sequence in all cases.

Results

The characterization of bovine MBP, C1

Its migration on SDS and acetic acid-urea slab, and alkaline-urea, disc gels was verified and the findings of Cheifetz et al. [26] confirmed. On HPLC, MBP, C1 eluted as a single peak (Fig. 1a).

The effects of heat and of pH 11

SDS and acetic acid-urea PAGE revealed no changes in migration of MBP, C1 following either pretreatment. However, alkaline-urea disc gel electrophoresis revealed diffuse streaking after heating and a significant retardation in migration after pH 11. Upon HPLC, the heat-pretreated MBP, C1 eluted as a somewhat rounded single peak (Fig. 1b) with minor additional absorbance noted immediately ahead of the main peak.

The carboxyl-^{3}H-methylation of MBP, C1

(A) Effect of [PM II]. Varying the concentration of PM II revealed the substoichiometric nature of the carboxyl-^{3}H-methylation reaction, methyl transfer to the native MBP, C1 not exceeding 5 pmol/h or 0.005 mol/mol of MBP, C1 at 32.5 μM PM II and 10 μM MBP, C1 (Fig. 2a). This amount increased more than

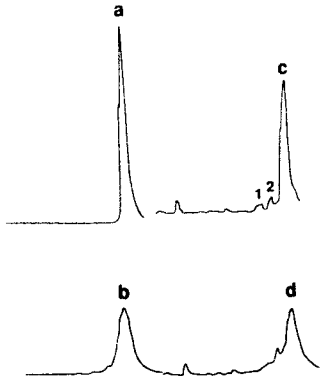

Fig. 1. The HPLC elution profile of bovine MBP, C1. (a) 20 μg of MBP, C1 dissolved in 200 μl of water were placed on a Vydac$_{18}$ column and were eluted under the conditions described in Methods; (b) as in a, except that, before HPLC, the sample containing MBP, C1 was heated at 100 °C for 45 min in an oil bath; (c) MBP, C1 was carboxyl-^{3}H-methylated in an assay system containing 10 μCi of *S*-adenosyl-L-[*methyl*-^{3}H]methionine (1 Ci/mmol) (20 μM), 5 μl of PM II (11.0 μM) and the following, at the final concentration: phosphate-citrate buffer (pH 6.0) 50 mM EDTA 0.4 mM and dithiothreitol 1.2 mM for 30 min at 37 °C. (d) as in c, except that MBP, C1 was first heat-pretreated as in b. HPLC was performed after the addition of 80 μl of 0.05% trifluoroacetic acid in water. The ordinate represents the A_{226} traces of MBP, C1 in arbitrary units. The areas labelled 1 and 2 in c are referred to in the text.

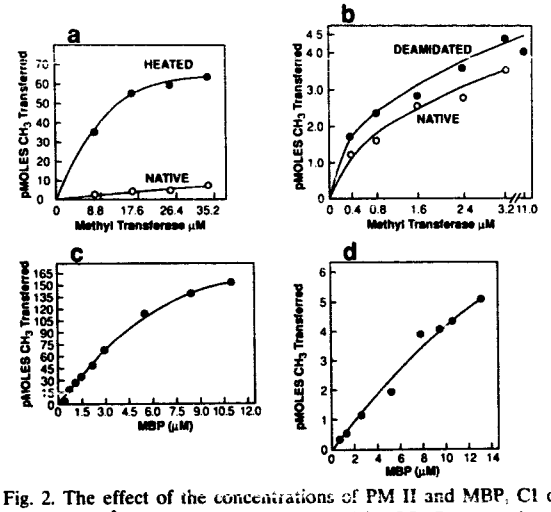

Fig. 2. The effect of the concentrations of PM II and MBP, C1 on the carboxyl-^{3}H-methylation of MBP, C1. (a) MBP, C1 was carboxyl-^{3}H-methylated for 60 min at 37 °C in the native state (○———○) and after a heat-pretreatment (see Fig. 1b) (●———●) in the presence of increasing concentrations of PM II (M_r 25000); (b) MBP, C1 was first exposed to pH 11.0 for 3 h at 37° (●———●), while the control (○———○) was co-incubated at pH 5.0; both were then carboxyl-^{3}H-methylated as in a; (c) MBP, C1 was first heat-pretreated and then carboxyl-^{3}H-methylated as in a in the presence of 11 μM PM II;(d) MBP, C1 was first exposed to alkali as in b and was then carboxyl-^{3}H-methylated as in a.

TABLE I

The carboxyl-^{3}H-methylation of peptide '1856' and of human brain myelin basic protein

Values are in pmol of methyl group transferred to MBP or peptide '1856'. Peptide '1856': H-Gly-Ser-Leu-Pro-Gln-Lys-Ser-Gln-Arg-Ser-Gln-Asp-Glu-Asn-Gly-OH. 36 μg of peptide and 17 μg of MBP were incubated for carboxyl-^{3}H-methylation in the presence of 11.8 μM PM II for 30 min at 37°C.

Pre-treatment	Peptide '1856'		MBP	
	pmol	x-fold	pmol	x-fold
None	0.37	1	5.84	1
(a) Heat (100°C), 10 min.	2.64	7.13	23.7	4.06
(b) pH 11.0, 3h, 37°C	2.92	7.89	6.31	1.06
(c) a+b	9.32	25.1	30.3	5.19
(d) b+a	5.33	14.4	24.2	4.14

10-fold when heat-pretreated MBP, C1 was carboxyl-^{3}H-methylated. The relatively minor effect of alkaline exposure is shown in Fig. 2b.

(B) Effect of [MBP, C1]. The dependence of the carboxyl-^{3}H-methylation of heat-pretreated (Fig. 2c) and alkali-exposed (Fig. 2d) MBP, C1 on its concentration revealed a curvilinear relationship between methyl transfer and concentration, with a greater tendency toward a carboxylmethylation plateau in the former case.

(C) Effect of combined heat and alkaline treatments. Table I illustrates the comparative effects of a brief (10 min) heat pretreatment and of a 3 h alkaline exposure, alone and in combination, on a synthetic pentadecapeptide (see Materials), containing one aspartate and one asparagine residue/mol in a sequence spanning discontinuously residues 70–76 and 80–83 of human MBP, and on total MBP (rather than the C1 charge isoform), purified [18] from the white matter of the brain of a 24-year-old male fatal accident victim. As expected, the short heat-pretreatment enhanced the carboxyl-^{3}H-methylation of MBP by 4.1-fold, while exposure to pH 11 had a minor effect; conversely, each pretreatment alone had a similar quantitative effect on the carboxyl-^{3}H-methylation of the peptide, while combining the pretreatments as in Table I, d resulted in an additive effect on the peptide, but not on MBP. Alternatively, under the condition in Table I, c there appeared to be an additive effect on the protein, but not on the peptide, the carboxylmethylation of the latter reaching much higher than additive levels.

(D) Effects on the structural integrity of MBP, C1. Native MBP, C1 was carboxyl-^{3}H-methylated at 37 °C and the reaction mixture was subjected to HPLC (Fig. 1c). SDS-PAGE [23] of samples taken from areas 1 and 2 and from under the main peak revealed a single band for the latter, which co-migrated with native MBP, C1. Area 1 contained a single band, migrating faster than MBP, C1 while area 2 contained some contaminating

MBP, C1 plus additional impurities, possibly breakdown products. Analogous findings were obtained when heat pretreated + carboxyl-^{3}H-methylated MBP, C1 (Fig. 1d) was similarly electrophoresed. These experiments showed minimal structural damage wrought upon MBP, C1 by virtue of its carboxyl-^{3}H-methylation with or without a prior heat-treatment (Fig. 1c,d). Since the assessment of structural damage was the primary purpose of these survey experiments, we did not determine whether any radioactivity associated with areas 1 and 2 of Fig. 1c.

The proteolytic fragmentation of MBP, C1

(A) With non-sequencing (NS) grade S. aureus V_8 proteinase. Native (cold) and carboxyl-^{3}H-methylated (radiolabelled) MBP, C1 were digested with (NS) V_8 at pH 4.0 and the digests analyzed by SDS-PAGE and HPLC. Several conditions were tested in order to make sure that MBP, C1 was totally digested. This was essential as, upon HPLC, some MBP, C1 fragments were found to co-migrate with the intact protein (data not shown). As illustrated in Fig. 3a (lanes 3 and 4), digestion had not ceased after 4 h at 37 °C, although no MBP, C1 was detectable. After 20 h, no MBP, C1 fragment larger than 14 kDa was present. Fig. 3b confirms that at 30 ° C, MBP, C1 was digested to completion only if 5 μg of the proteinase and 16 h of digestion were used (lane 5). Increasing the temperature to 37 °C failed to compensate for a shorter incubation time (lane 2) or for less proteinase (lane 8). The failure of (NS) V_8 (5 μg, 30 °C, 8.5 h) to cleave radiolabelled MBP, C1 to fragments smaller than 14 kDa is also apparent (lane 10).

(B) With sequencing (S) grade S. aureus V_8 proteinase. The differences in digestibility between the native and the radiolabelled MBP, C1, alluded to above, became even more evident when 1 μg of (S) V_8 was used to digest the protein. Fig. 3c clearly shows that digestion of the radiolabelled protein lagged behind that of the native species (compare lane 3 to lane 5) and that digestion of the heat-pretreated, native MBP, C1 proceeded more efficiently than that of the corresponding radiolabelled protein (compare lane 4 to lane 2). These differences were reproduced, although in a less striking manner, using HPLC (Fig. 4). Clearly the A_{226} profile of the native MBP, C1 digest (Fig. 4a) was simpler than that of the radiolabelled digest (Fig. 4b) in the late portion of the gradient (36–38 min). The profiles derived from the heat-pretreated native and radiolabelled MBP, C1 (Fig. 4c and d) reflected an extra peak at 40.04 min in the former profile. The radioactivity profiles (Fig. 5) revealed a specific and large effect of heat on the labelling of the fragment eluting at 36.5 min. Because peptides from this region of the gradient were not available in a state of sufficient purity, their partial amino acid sequence could not be determined. Finally, when MBP, C1 was carboxyl-^{3}H-methylated after exposure to pH 11 and was then digested by (S) V_8, the HPLC absorbance profiles were similar to those shown in Fig. 4. Since the la-

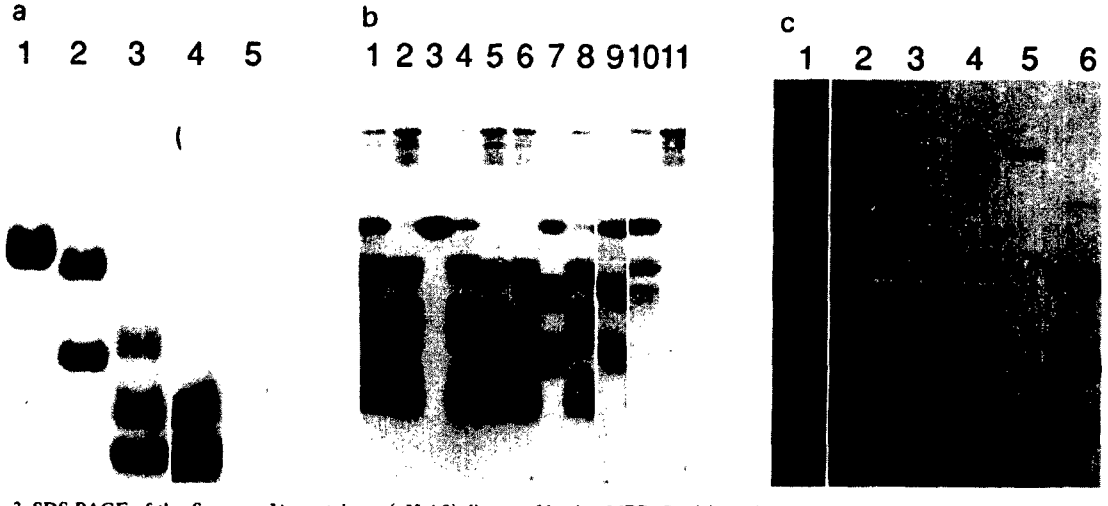

Fig. 3. SDS-PAGE of the *S. aureus* V_8 proteinase (pH 4.0) digests of bovine MBP, C1. (a) 1: 10 μg C1; 2: molecular weight standards: soybean trypsin inhibitor (20100) and α-lactalbumin (14200); 3: 4 h, 37 °C, 5 μg (NS) V_8; 4: 20 h, 37 °C, 5 μg (NS) V_8; 5: 5 μg (NS) V_8; (b) 1: 8 h, 37°C, 2.5 μg (NS) V_8; 2: as 1, 5 μg (NS) V_8; 3: 10 μg C1; 4: 16 h, 30 °C, 2.5 μg (NS) V_8; 5: as 4, 5 μg (NS) V_8; 6: as 4, 4 μg (NS) V_8; 7: molecular weight standards, as in a, 2 + aprotinin (6500); 8: 16 h, 37 ° C, 2.5 μg (NS) V_8; 9: as in 7; 10: radiolabelled C1 digest, 8.5 h, 30 °C, 5 μg (NS) V_8; 11: 5 μg (NS) V_8; (c) 1: 10 μg C1; 2–5: digests of 2: heat-pretreated (30 min, 100 °C), carboxyl-^{3}H-methylated C1; 3: native, carboxyl-^{3}H-methylated C1; 4: heat-pretreated (30 min, 100 °C) C1; and 5: native C1; 6: molecular weight standards. In (c), the digests were generated by 1 μg (S) V_8, 20 h at 30 °C.

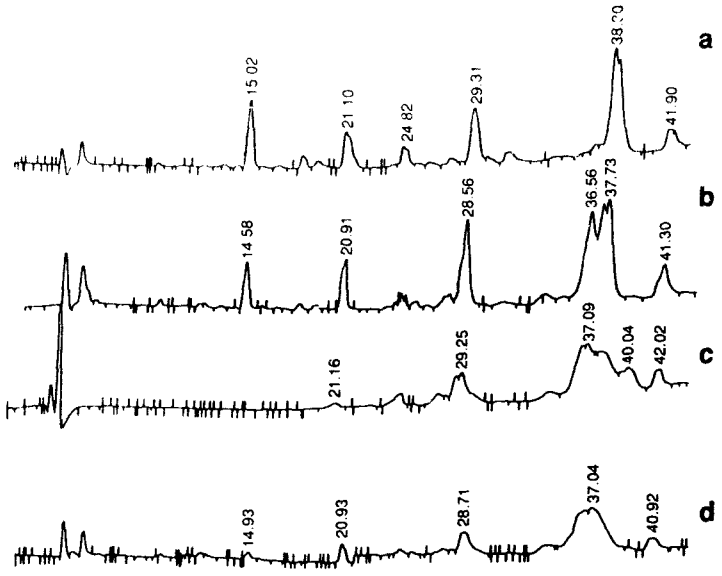

Fig. 4. HPLC profiles of *S. aureus* V_8 proteinase digests of bovine MBP, C1. Native MBP, C1 was digested (a) directly, (b) after carboxyl-^{3}H-methylation, (c) after heat pretreatment (15 min, 100 °C) and (d) after heat pretreatment and carboxyl-^{3}H-methylation. Radiolabelled MBP, C1 (b and d) was separated from the carboxylmethylation assay reactants, as described in Methods. All MBP, C1 samples were lyophilized before suspension in 25 μl of freshly prepared 40 mM ammonium acetate (pH 4.0) and digestion with 1 μg of (S) V_8 for 20 h at 30 °C HPLC was as described in Methods. Absorbance was monitored at 226 nm. The peak at 40.9–42.0 min represents (S) V_8 protein. SDS-PAGE of all digests revealed no residual MBP, C1. The numbers above the peaks mark elution time in min.

belling profiles of the native and the alkali-exposed MBP, C1 fragments were within experimental error, they are not shown.

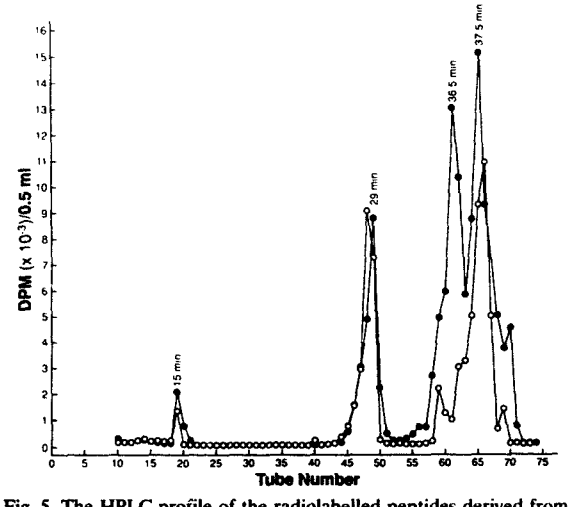

Fig. 5. The HPLC profile of the radiolabelled peptides derived from *S. aureus* V_8 proteinase digestion of carboxyl^{3}H-methylated native and heat-pretreated MBP, C1. 40 μg of native (○——○) and heat-pretreated (●——●) MBP, C1 (30 min, 100 °C) were carboxyl-^{3}H-methylated (60 min, 37 °C) the radiolabelled MBP, C1 was re-isolated, lyophilized and one half of each of the two suspended residues (each containing in excess of $1 \cdot 10^6$ dpm) was digested by (S) V_8 (1 μg) for 18 h at 30 °C. The other half was subjected to SDS-PAGE (Fig. 3c). The digests were separated by HPLC and the radioactivity of the fractions was determined.

(C) With trypsin. (1) Carboxyl-^{3}H-methylated MBP, C1. The digestion was for 40 min at 37 °C and pH 6.0 and the fragments were separated by HPLC under the conditions described in Ref. 9. The most highly radiolabelled of the eight fragments (data not shown) eluted at 20.5 min; there was no significant radioactivity beyond 32 min, i.e., in the tryptic fragment previously stated to contain Asp-82 and Asn-84 of human MBP, C1 [9].

2. Carboxyl-^{3}H-methylated (S) V_8 fragment P_{29}. Tryptic subdigestion of carboxyl-^{3}H-methylated (S) V_8 peptide P_{29} (tubes 46–49, Fig. 5, open circles) yielded six radiolabelled peptides, one of which, No. 2 (Fig. 6a), was partially sequenced (see below), together with the predominant unlabeled fragment, eluting at 15.18 min (Fig. 6b). In accordance with similar findings by others [7], we also noted perceptible temporal differences in elution between a given native vs. carboxyl-^{3}H-methylated tryptic fragment. This was particularly apparent in the case of the radiolabelled peptide 2 (Fig. 6a) collected as a peak in tube 26 (i.e., at 13 min) as against its native counterpart, which eluted with a peak at 12.27 min (Fig. 6b).

The identification of the peptides derived from native MBP, C1

The use of *S. aureus* V_8 proteinase at pH 4.0 to cleave human MBP, C1 has been reported to generate three peptides [16] with presumed sequences matching residues 1–83, 84–119 and 120–170. It should be noted

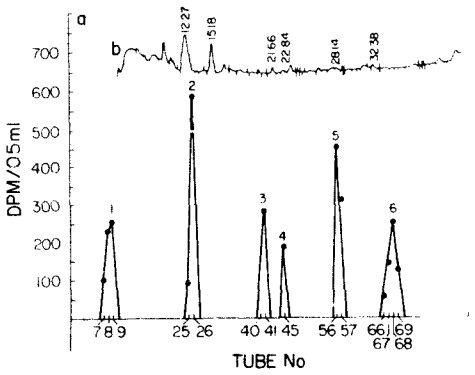

Fig. 6. The tryptic subdigestion of *S. aureus* (S) V_8 proteinase peptide (P_{29}). MBP, C1 (20 μg) was digested with 1 μg of (S) V_8 proteinase in the presence of 25 μl of freshly prepared 50 mM ammonium acetate (pH 4.0) in a final volume of 40 μl. Six identical tubes were incubated for 18 h at 30 °C and the digests pooled and placed on a Vydac C_{18} column. Separation of the fragments and the isolation of P_{29} was as described in Methods and in the legend to Fig. 4. Radiolabelled P_{29} was obtained similarly from 20 μg of carboxyl-^{3}H-methylated MBP, C1 (see legend to Fig. 4, condition b). Native and carboxyl-^{3}H-methylated P_{29} were combined in a 3:1 (w/v) ratio and to each of four aliquots of this mixture, containing 2 nM P_{29} in 25 μl of 0.2 M sodium citrate (pH 6.0), 2 μg of trypsin were added. Incubation was for 40 min at 37 °C. Separation of the tryptic digest was according to Ref. 9. The radioactivity profile of the digest is shown in a and the A_{226} nm profile is shown in b. Peak 1 in a most likely represents non-peptide radioactivity which was lost from the carboxyl-^{3}H-methylated P_{29} during incubation and/or the subsequent workup. Peak 2 was subjected to partial amino acid sequencing (see Results), alongside with the 15.18 min absorbance peak, shown in b.

that no direct evidence warranting such a conclusion was ever produced, either in 1987 [16] or subsequently [27]. In our hands, the cleavage of bovine MBP, C1 at pH 4.0 yielded the A_{226} profile shown in Fig. 4a. In an effort to determine the structure of the fragment eluting at 29.31 min (P_{29}) (taken to correspond to peptide I of Ref. 16), we subjected it to six cycles of N-terminal amino acid analysis. The unexpected findings invalidate the conclusion [16] that this peptide comprises residues 82–118, given that the six amino acids sequenced: Gly-Arg-Ala-Ser-Asp-Tyr, a match of residues 128–133, following cleavage at Gly-127–Gly-128. Partial sequencing of the 38.3 min fragment (Fig. 4a) yielded Ser-Leu-Gly-Arg-Phe- a match of residues 38–42 and evidence of cleavage at Asp-37–Ser-38.

We also determined the partial sequence of the peptide eluting at 21.10 min (Fig. 4a) to be: Thr-Gly-Ileu-Leu, a match of residues 33–36 resulting from cleavage at Asp-32–Thr-33.

The identification of the peptides derived from carboxyl-^{3}H-methylated MBP, C1

Cleavage of carboxyl-^{3}H-methylated MBP, C1 at pH 4.0 resulted in the A_{226} and radioactivity profiles shown in Figs. 4b and 5 (open circles). Partial sequencing of the radiolabelled fragment eluting at 28.56 min (P_{29}) (Fig. 4b) revealed the sequence Gly-Tyr-Gly-Gly-Arg (125–129), evidence of cleavage at Phe-124–Gly-125, 3 residues (Gly-125, Tyr-126 and Gly-127) closer to the N-terminus of MBP, C1 than in the native protein. Since the peptide eluting at 14.58 min (Fig. 4b) sequenced as Gly-Phe-Gly-Tyr-Gly (123–127), the following minimal structure (123–132) in which Asp-132 is modified (isomerized or racemized) and hence carboxyl-^{3}H-methylated, is proposed for it: Gly-Phe-Gly-Tyr-Gly-Gly-Arg-Ala-Ser-Asp. Confirmation of these (S) V_8 proteinase cleavage sites was obtained by tryptic subdigestion of P_{29} (Fig. 6). Of the six radiolabelled peptides eluting upon HPLC [9], the 'most radioactive' one was partially sequenced as Gly-His-Asp (142–144) (Fig. 6a, peak No. 2), while the only prominent 'unlabeled' fragment, eluting at 15.18 min (Fig. 6b) analyzed as Ileu-Phe (152–153). These findings thus point to the following, probably still incomplete structure for the carboxyl-^{3}H-methylated peptide P_{29}: Gly-Tyr-Gly-Gly-Arg-Ala-Ser-Asp-Tyr-Lys-Ser-Ala-His-Lys-Gly-Leu-Lys-Gly-His-Asp-Ala-Gln-Gly-Thr-Leu-Ser-Lys-Ileu-Phe-Lys-, a match of residues 125–154 of bovine MBP, C1.

The localization of the carboxylmethylatable sites in MBP, C1

The evidence derived from the proteolysis experiments suggests that several L-aspartates of MBP, C1 exist as carboxylmethylatable sites. While one of these, Asp-132, appears to be contained within peptides $P_{14.5}$ and P_{29}, another, Asp-144, resides most likely only within P_{29}. Preliminary evidence obtained from the partial sequencing of native fragment 38.3 (Fig. 4a) points to Asp-46 as yet another carboxylmethylatable site. The definitive confirmation of this and, possibly, of additional putative methyl-accepting modified L-aspartate residues in MBP, C1 must await an improved separation of the late-eluting (S) V_8 fragments. This also appears essential before the heat-reactive L-aspartates responsible for the increase in carboxylmethylation of MBP, C1 (Fig. 5, full circles, 36.5 min peak) may be identified.

Discussion

Although the carboxylmethylation of MBP has been documented previously [14,23] and the initial rates of this reaction were found to be significantly different among four charge isoforms of bovine MBP [15], the factors and conditions governing the interaction of PM II with the methyl-accepting sites within the protein have heretofore not been examined. In a previous brief report from our laboratory [28], it was noted that the carboxylmethylation of MBP increases significantly af-

ter its heat pretreatment, carried out in an unbuffered aqueous solution (pH 5–6) while, conversely, only a minimal increment in this process was observed after its exposure to alkaline (pH 11.0) conditions at 37 °C for 3h. These findings, validated by the results shown in Fig. 2, infer that the L-aspartate residues of MBP, C1 convert into L-isoaspartate more readily than do its L-asparagine residues, albeit under admittedly non-physiological conditions. Since, under alkaline conditions, L-asparagine converts to L-isoaspartate most readily when next to glycine [29,30] a bond absent in MBP, the refractoriness of Asn-83 and Asn-91, (linked respectively to Pro-84 and Ileu-92), to generate L-isoaspartate is readily understood.

For the identification of the methyl-accepting sites of bovine MBP, C1 we relied on the report [16] alleging (NS) V_8 trisection at Glu-83–Asn-84 and Glu-119–Gly-120. Initially, we also used (NS) V_8 to fragment MBP, C1, but as shown by the SDS-PAGE banding profile of the digest, trisection failed to occur (Fig. 3a and b). At 30 °C, under conditions assuring total digestion of the protein (Fig. 3b, lanes 5 and 6), no fewer than four to five fragments were observed. When radiolabelled MBP, C1 was digested (Fig. 3b, lane 10), proteolysis appeared greatly retarded. SDS-PAGE of an (S) V_8 digest (Fig. 3c) confirmed this refractoriness (compare lanes 3 and 5).

The examination of the effect of the heat pretreatment on the subsequent action of (S) V_8 proteinase revealed a slowdown in its proteolysis. Yet if the heat-pretreated MBP, C1 was first carboxyl-^{3}H-methylated and then fragmented, no slowdown of this process was discernible (Fig. 3c, lanes 2 and 3). This observation suggests that the heat pretreatment of MBP, C1 and its subsequent carboxylmethylation affect the action of (S) V_8 proteinase non-additively and in a rather complex way. Further evidence in favor of this notion was provided by the appearance of a novel fragment (40.04 min) in digests derived from heat-pretreated MBP, C1 (Fig. 4c) at the apparent expense of the virtual disappearance of the 14.5–15 min fragment, seen in all the other digests (Fig. 4a, b and d).

The assumption which prompted the use of *S. aureus* V_8 proteinase at pH 4.0 was based on the notion that this would produce the three peptides 1–82, 83–118 and 119–169. The present findings invalidate this assumption, as the proteinase cleaved native MBP, C1 at Gly-127–Gly-128, Asp-32–Thr-33 and Asp-37–Ser-38 and radiolabelled (carboxylmethylated) MBP, C1 at Phe-124–Gly-125 and Pro-122–Gly-123. Although, at present, this unorthodox and non-specific cleavage of MBP, C1 has no cogent explanation, the qualitatively different fragmentation patterns of native vs. radiolabelled MBP, C1 (Figs. 3 and 4) may be the result of ^{3}H-methyl groups esterifying the carboxyls of modified aspartates-132 and 144 in the radiolabelled protein with a consequent diminished access of the proteinase to its target sites and re-direction of cleavage to a more accessible site upstream (toward the N-terminus).

Our findings reveal that at pH 4.0 in 40 mM freshly prepared ammonium acetate, the sequencing grade *S. aureus* V_8 (Boehringer) cleaved bovine MBP, C1 not as the enzyme hitherto relied upon to cleave Glu-X bonds in proteins [31–33], but rather as a non-specific acid proteinase. Originally, the inclusion of 2 mM EDTA in *S. aureus* V_8 proteinase incubates was recommended (but not prescribed) [33] to insure inhibition of a presumed, contaminating neutral proteinase. It was equally stated at that time, however, that such a neutral proteinase "does not normally contaminate the glutamyl-specific enzyme preparations" [33], i.e., those incubating at pH 4.0 in ammonium acetate [31]. For this reason and others, enumerated below, we rule out cleavage of MBP, C1 by such a contaminating neutral proteinase. First, because proteolysis under such conditions would have had to yield some fragments resulting from orthodox *S. aureus* V_8 proteinase-catalyzed Glu-X cleavage, which were however, not detected, secondly because the HPLC profile of 20 μg of the commercial *S. aureus* V_8 proteinase (sequencing grade, see Materials) indicated absolutely no A_{215} nm absorbing impurity and thirdly, because at pH 4.0, a contaminating neutral proteinase would not be expected to match in quantity the number of fragments resulting from the action of the majority ingredient of the commercial preparation sold as sequencing grade *S. aureus* V_8 proteinase, namely the V_8 proteinase itself. Lastly, we checked the pattern of insulin B_{ox} cleavage by the Boehringer product at pH 4.0 and found it to be identical to that generated at pH 7.8 (see Materials). Hence, we see no valid reason for the assumption that the fragments noted after proteolysis of MBP, C1, such as reported in this paper, are not the exclusive product of *S. aureus* V_8 proteinase action. A recent case of similar apparent deviation of this proteinase from orthodox and exclusive cleavage of Glu-X bonds is its reported cleavage (during a 21-h incubation at 37 °C, in 50 mM phosphate (pH 7.8) and 2 mM EDTA) of Gly-Asn, Gly-Arg, Arg-Asn and Lys-Ala bonds in the 'low molecular weight' acid phosphatase of ox liver [34]. In this study the proteinase also failed to cleave one of the Glu-X bonds present, namely Glu_{128}–Asp_{128}, where Asp is linked to Pro. More recently, Banik et al. [35] reported that a Ca^{2+}-activated proteinase cleaves human MBP, C1 at Arg-49–Gly-50, Arg-65–Thr-66, Phe-89–Phe-90, Val-95–Thr-96, Gly-121 and Gly-164–Ser-165. It thus appears that when certain proteins possess a number of *S. aureus* V_8 proteinase-resistant Glu-X bonds, such as Glu-Asp, Glu-Asn and Glu-Gly, where X is, respectively, linked to Pro (liver acid phosphatase [34] and bovine MBP, C1, this paper) and Gln (bovine MBP, C1, this paper)

and under a set of conditons, both acidic and neutral, yet to be fully defined, *S. aureus* V_8 proteinase behaves as a non-specific proteinase [35,36], rather than as the well-known and trusted Glu-X cleaving proteinase.

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