Purification and Properties of Different Forms of Modeccin, the Toxin of *Adenia digitata*

SEPARATION OF SUBUNITS WITH INHIBITORY AND LECTIN ACTIVITY

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1. The subunits were isolated of modeccin (subsequently referred to as modeccin 4B), the toxin purified from the roots of *Adenia digitata* by affinity chromatography on Sepharose 4B [Gasperi-Campani, Barbieri, Lorenzoni, Montanaro, Sperti, Bonetti & Stirpe (1978) *Biochem. J.* 174, 491–496]. They are an A subunit (mol.wt. 26 000), which inhibits protein synthesis, and a B subunit (mol.wt. 31 000), which binds to cells. Both subunits, as well as intact modeccin, gave single bands on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, but showed some heterogeneity on iso-electric focusing and on polyacrylamide-gel electrophoresis at pH 9.5. 2. A second form of modeccin, not retained by Sepharose 4B, was purified by affinity chromatography on acid-treated Sepharose 6B: this form is subsequently termed modeccin 6B. 3. Modeccin 6B has a molecular weight indistinguishable from that of modeccin 4B, and consists of two subunits of mol.wts. 27 000 and 31 000, joined by a disulphide bond. The subunits were not isolated because of their high insolubility in the absence of sodium dodecyl sulphate. 4. As compared with modeccin 4B, modeccin 6B is slightly less toxic to animals, does not agglutinate erythrocytes, and is a more potent inhibitor of protein synthesis in a lysate of rabbit reticulocytes, giving 50% inhibition at the concentration of 0.31 µg/ml.

Modeccin, the potent toxin of *Adenia digitata* (Refsnes *et al.*, 1977; Stirpe *et al.*, 1978), is a protein with mol.wt. approx. 60 000 (63 000, Olsnes *et al.*, 1978; 57 000, Gasperi-Campani *et al.*, 1978), which inhibits protein synthesis in cells and in cell-free systems, acting on the 60S ribosomal subunit *in vitro* (Montanaro *et al.*, 1978; Olsnes & Abraham, 1979) as well as *in vivo* (Sperti *et al.*, 1979), and making it unable to bind elongation factor 2.

This toxin was purified by affinity chromatography on immobilized concanavalin A (Refsnes *et al.*, 1977) or desialylated fetuin (Olsnes *et al.*, 1978) or on Sepharose 4B (Gasperi-Campani *et al.*, 1978). With the latter procedure it was observed that the material not retained by Sepharose 4B inhibited protein synthesis by a lysate of rabbit reticulocytes to a greater extent than did modeccin, although it was somewhat less toxic to animals.

Modeccin prepared in our laboratory, subsequently referred to as modeccin 4B, gave a single

band on SDS/polyacrylamide-gel electrophoresis. Treatment with 2-mercaptoethanol brought about significant changes in the toxin molecule in that (i) two distinct bands were observed on SDS/polyacrylamide-gel electrophoresis, (ii) the inhibitory effect on cell-free protein synthesis was increased, and (iii) the toxicity to animals was decreased (Gasperi-Campani *et al.*, 1978). Similar results were obtained by Olsnes *et al.* (1978). It was concluded that modeccin consists of two different subunits, joined together by at least a disulphide bond. The subunits could not be purified in separate forms (Gasperi-Campani *et al.*, 1978; Olsnes & Abraham, 1979). However, by analogy with the related toxins ricin and abrin (review by Olsnes & Pihl, 1977), it was supposed that they could be a subunit A (for activity), responsible for the inactivation of ribosomes, and a subunit B (for binding), capable of binding to galactosyl receptors on the cell surface.

In the experiments reported in the present paper the material that inhibits protein synthesis was purified from the effluent not retained by Sepharose

Abbreviation used: SDS, sodium dodecyl sulphate.

4B, and was identified as another form of modeccin, subsequently referred to as modeccin 6B, since it binds to acid-treated Sepharose 6B. Further, the subunits of modeccin 4B were isolated, and evidence was obtained that they are an A and a B subunit, with the expected properties described above.

Experimental

Materials

Roots of *Adenia digitata* were obtained from the Department of Botany, University of Pretoria, from the Botanical Research Institute, Pretoria, Republic of South Africa, and from the National Herbarium, Salisbury, Rhodesia. Modeccin was purified as described by Gasperi-Campani *et al.* (1978).

L-[^{14}C]Leucine (specific radioactivity 342 mCi/mmol) and *N*-ethyl[2,3- ^{14}C]maleimide (specific radioactivity 4.7 mCi/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Sepharose 4B, Sepharose 6B and Sephadex G-100 (superfine grade) were from Pharmacia Fine Chemicals, Uppsala, Sweden; acid-treated Sepharose 6B was prepared as described by Ersson *et al.* (1973) with a 3 h acid hydrolysis. Markers for molecular-weight determinations were obtained from Boehringer Mannheim G.m.b.H., Mannheim, West Germany. Neuraminidase from *Vibrio comma* was purchased from Behringwerke, Marburg/Lahn, West Germany (one unit is defined as the amount of enzyme required to release 1 μg of *N*-acetylneuraminic acid from human α_1 -acid glycoprotein in 15 min at 37°C), and bovine serum albumin from Sigma Chemical Co., St. Louis, MO, U.S.A. All other chemicals were from the same sources as described by Gasperi-Campani *et al.* (1978).

Haemagglutinating activity

This was determined in Greiner micro-titre plates, U-shaped (Microcult M-29, Pool Bioanalysis Italiana, Milan, Italy), with pig erythrocytes formalin-treated as described by Butler (1963). Each well contained, in a final volume of 100 μl : the preparations to be tested, in serial dilutions by doubling, 2.5 units of neuraminidase, and 50 μl of a 1.2% (v/v) suspension of formalin-treated pig erythrocytes. All solutions and suspensions were in 0.14 M-NaCl containing 20 mM-sodium phosphate buffer, pH 7.2, and bovine serum albumin (15 $\mu\text{g}/\text{ml}$). Addition of neuraminidase was essential, since without the enzyme agglutination was obtained with freeze-dried modeccin 4B, but not with the freshly prepared toxin. Haemagglutination was evaluated visually after at least 1 h at room temperature (20°C).

Protein synthesis

Protein synthesis was determined as described previously (Gasperi-Campani *et al.*, 1978) with a lysate of rabbit reticulocytes prepared as described by Allen & Schweet (1962). Reaction mixtures contained, in a final volume of 0.25 ml: 10 mM-Tris/HCl buffer, pH 7.4, 100 mM-ammonium acetate, 2 mM-magnesium acetate, 1 mM-ATP, 0.2 mM-GTP, 15 mM-phosphocreatine, 12 μg of creatine kinase, 0.05 mM-amino acids (minus leucine), 0.75 μCi of L-[^{14}C]leucine and 0.1 ml of lysate. Incubation was at 27°C for 5 min. Radioactivity incorporated into protein was determined in 25 μl samples as described by Gasperi-Campani *et al.* (1978). The inhibitory activity of the preparations tested was expressed as ID₅₀ (concentration giving 50% inhibition) and was calculated by the linear-regression method.

Polyacrylamide-gel electrophoresis and molecular-weight determinations

One-dimensional electrophoresis on polyacrylamide gels was performed at pH 4.5 in β -alanine/acetic acid buffer as described by Reisfield *et al.* (1962) and at pH 9.5 in sodium glycinate buffer with gels containing 7% polyacrylamide as described by Liao *et al.* (1969). SDS/polyacrylamide-gel electrophoresis at pH 7.0 was carried out in gel columns as described by Weber & Osborn (1969); discontinuous SDS/polyacrylamide-gel electrophoresis in gel slabs (14 cm $\times$ 10 cm $\times$ 1.5 mm thick) was performed with the Laemmli (1970) system. Two-dimensional electrophoresis was carried out by the method of O'Farrell (1975), in which proteins are separated according to charge in the first dimension and according to molecular weight by electrophoresis in the presence of SDS in the second dimension. Separation by charge was obtained either by gel electrophoresis at pH 9.5 (Liao *et al.*, 1969) or by isoelectric focusing with 2% Ampholines, pH range 3.5–10, as described by Catsimpoolas (1968). In both cases samples were run in duplicate: at the end of the run one of the gels was stained, and the second was extruded into 5 ml of 0.0625 M-Tris/HCl buffer, pH 6.8, containing 10% (w/v) glycerol and 2.3% (w/v) SDS. After equilibration at room temperature for 2 h, the gel either was stored frozen at –80°C or was immediately loaded on the second-dimension gel (O'Farrell, 1975). Second-dimension electrophoresis in the presence of SDS was carried out in the model 220 Vertical Slab Electrophoresis cell (Bio-Rad Laboratories, Richmond, CA, U.S.A.) with the Laemmli (1970) system as described in the instruction manual. Isoelectric-focusing gels were stained with 0.1% Coomassie Blue in 50% (w/v) trichloroacetic acid for 20 min, destained with several changes of 40% (v/v) methanol at 50°C, and finally stored in 7.5% acetic acid. Gel columns and slabs

from which Ampholines were absent were stained and destained as described by Weber & Osborn (1969).

Molecular-weight determinations by thin-layer gel filtration were performed on Sephadex G-100 (superfine grade), with as solvents 0.5M-NaCl (Morris, 1964), 0.02M-sodium phosphate buffer, pH 7 (Johansson & Rymo, 1964), or 0.12M-sodium glycinate buffer, pH 9.5.

Determination of thiol groups

Protein samples (3–25 μ g) in 100 μ l of 50mM-Tris/HCl buffer, pH 8.0, were mixed with 20 μ l of 0.8mM-*N*-ethyl[14 C]maleimide, and were incubated at 37°C for 2h in either the absence or the presence of 8M-urea. At the end of the incubation protein was precipitated with 10% (w/v) trichloroacetic acid, 50 μ g of albumin was added, and the precipitate was collected on Whatman GF/C filters, washed with 5% trichloroacetic acid and counted for radioactivity. In all calculations the functional specific radioactivity of *N*-ethyl[14 C]maleimide (7d.p.m./pmol) was used, and was determined (Steinert *et al.*, 1974) by titrating yeast alcohol dehydrogenase, which has 8 thiol groups per molecule.

Other determinations

Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard, or spectrophotometrically (Kalb & Bernlohr, 1977).

Radioactivity was determined with 5ml of methoxyethanol and 10ml of scintillation fluid [0.05% 1,4-bis-(5-phenyloxazol-2-yl)benzene and 0.4% 2,5-diphenyloxazole in toluene] in a Packard Tri-Carb liquid-scintillation spectrometer with an external standard. Counting efficiency was better than 80% for 14 C.

Radioactivity in polyacrylamide gels was measured in sections of the gel placed in counting vials with 0.2ml of 36% (v/v) H_2O_2 for 5h at 50°C; 0.4ml of Soluene-350 (from Packard Instrument Co., Downers Grove, IL, U.S.A.) was then added and the samples were kept overnight at room temperature before the addition of the scintillation fluid.

Results

Purification of modeccin 6B

All operations were performed at 2–4°C. Roots of *Adenia digitata* were minced and were homogenized in a blender with 5 vol. of phosphate-buffered saline (0.2M-NaCl in 0.005M-sodium phosphate buffer, pH 7.2), containing 0.1M-cystamine hydrochloride previously neutralized with NaOH. After overnight stirring, the suspension was filtered through filter paper on a Büchner funnel, the filtrate

Fig. 1. Purification of modeccin 4B by affinity chromatography

The precipitate obtained at 65% saturation of $(\text{NH}_4)_2\text{SO}_4$ (203mg of protein), dissolved in and dialysed against phosphate-buffered saline, was applied to a column (90cm $\times$ 1.5cm) of Sepharose 4B, previously equilibrated with the same solution. After washing, the column was eluted with 0.2M-galactose (arrow) in phosphate-buffered saline. Fractions (2.5ml) were collected at 12 ml/h.

was clarified by centrifugation at 11000g for 20min, and was adjusted to 65% saturation with solid $(\text{NH}_4)_2\text{SO}_4$, added slowly under constant stirring. The precipitate was collected by centrifugation, dialysed against phosphate-buffered saline (without cystamine) and applied to a column of Sepharose 4B. Under particularly good resolving conditions three peaks emerged with the effluent and washings, and another peak (modeccin 4B) was eluted from the column with 0.2M-galactose (Fig. 1).

The material of peak III of the effluent had a strong inhibitory activity on protein synthesis. The fractions of this peak were pooled, concentrated in a macro-solute concentrator (Minicon; Amicon, Lexington, MA, U.S.A.), and 5.4mg of protein was applied to a column (80cm $\times$ 1cm) of acid-treated Sepharose 6B. A single peak (modeccin 6B) was obtained on elution of the column with 0.2M-galactose. The preparation of modeccin 4B and of modeccin 6B is summarized in Table 1.

It should be pointed out that the experimental conditions of Fig. 1 gave exceptionally good resolution of the peaks of material not retained by Sepharose 4B, and actually the concentrated material of peak III gave a major electrophoretic band in the same position as modeccin 6B, with only a slight contamination by a faster band (see Fig. 2). Further, passage of this material through Sepharose 6B caused some loss of the specific inhibitory activity, presumably owing to partial degradation of the protein. However, usually we employed larger and more heavily loaded columns of Sepharose 4B, and with these the eluate was badly resolved (see

Table 1. *Purification of modeccin 4B and of modeccin 6B*

Crude extract was prepared from 200g of roots of *Adenia digitata*. Data for stages after $(\text{NH}_4)_2\text{SO}_4$ precipitation are corrected to this amount, and the quantities actually used are given in the legend to Fig. 1 and in the text. Protein synthesis was determined as described in the text, and the inhibitory activity is expressed as ID_{50} (concentration, in $\mu\text{g}/\text{ml}$, giving 50% inhibition).

Preparation	Total protein (mg)	Inhibitory activity on protein synthesis		
		Specific activity (number of ID_{50}/mg of protein)	Total activity ($10^{-3} \times$ number of ID_{50})	Yield (%)
Crude extract	9823	56	548	100
$(\text{NH}_4)_2\text{SO}_4$ precipitate	716	694	497	91
Sephacrose 4B				
Peak I	8	0	0	
Peak II	373	102	38	2
Peak III	46	4348	200	36
Eluate with 0.2 M-galactose (modeccin 4B)	90	397	36	6
Peak III, concentrated	22	7642	174	32
Sephacrose 6B				
Eluate with 0.2 M-galactose (modeccin 6B)	14	4000	56	10

Fig. 1 in Gasperi-Campani *et al.*, 1978). In these cases modeccin 6B could be separated by applying the pooled effluent to Sepharose 6B and eluting this column as described above.

Electrophoretic behaviour of modeccin 4B and of modeccin 6B

SDS/polyacrylamide-gel electrophoresis at pH 7.0 gave indistinguishable molecular weights for the two forms of modeccin (56000 ± 1000), and t.l.c. in all solvents used confirmed a molecular weight lower than that of bovine serum albumin for both native molecules, in the absence of SDS. However, the two forms of modeccin showed differences in (i) the molecular weight of the constituent subunits, (ii) the isoelectric point and (iii) the electrophoretic mobilities of the multiple bands obtained at pH 9.5 in the absence of SDS. A single band was instead observed on electrophoresis at pH 4.5.

Molecular weight of the constituent subunits. Both modeccin 4B and 6B on reduction with 2-mercaptoethanol were split into two subunits, which appeared as separate bands on SDS/polyacrylamide-gel electrophoresis (Fig. 2). However, the mobility of the larger subunit was practically the same for both forms of modeccin (mol.wt. 31000 from the calibration curve obtained with the appropriate markers), whereas the smaller subunit of modeccin 6B showed a lower mobility (mol.wt. 27000) than the smaller subunit of modeccin 4B (mol.wt. 26000). A third band with the same molecular weight as the smaller subunit of modeccin 4B (26000) appeared in the gels loaded with peak-III material of the unretained effluent from Sepharose 4B and on the gels heavily loaded with modeccin 6B, and might be due to a slight contamination with modeccin 4B.

Fig. 2. *SDS/polyacrylamide-gel electrophoresis of modeccins and subunits*

Discontinuous electrophoresis on gel slab was performed as described in the Experimental section. (a) Modeccin 4B reduced with 2-mercaptoethanol ($18 \mu\text{g}$ of protein); (b) modeccin 6B reduced with 2-mercaptoethanol ($15 \mu\text{g}$); (c) modeccin 6B reduced with 2-mercaptoethanol ($25 \mu\text{g}$); (d) unretained effluent from Sepharose 4B (peak III of Fig. 1) reduced with 2-mercaptoethanol ($25 \mu\text{g}$); (e) unretained effluent from Sepharose 4B (peak III of Fig. 1; $25 \mu\text{g}$); (f) modeccin 6B ($25 \mu\text{g}$); (g) isolated subunit A of modeccin 4B ($24 \mu\text{g}$); (h) isolated subunit B of modeccin 4B ($25 \mu\text{g}$). Migration was from cathode (top) to anode (bottom). The arrows indicate the Bromophenol Blue front.

Isoelectric points of modeccin 4B, of its subunits and of modeccin 6B. Modeccin 4B showed four major bands with pI values in the range 7.1–7.6. This pattern resembles that reported by Olsnes *et al.* (1978), who observed at least four isotoxins in their preparation of modeccin. After isolation of subunits (see below), subunit A showed three bands with pI 5.8, 6.2 and 6.7, and subunit B gave four

major bands with pI values between 7.3 and 8.2. In the isoelectric-focusing gel of modeccin 6B most of the material focused in the region of the gel with pH higher than 8.5.

Polyacrylamide-gel electrophoresis at pH 9.5. Modeccin 4B gave five clear bands in the middle part of the gel with mobilities (distance of protein migration/distance of Bromophenol Blue migration) between 0.40 and 0.63, whereas most of the material of modeccin 6B remained in the upper part of the gel (mobility between 0.15 and 0.20). After reduction with 2-mercaptoethanol at pH 8.5, modeccin 4B gave seven bands with mobilities distinct from those of the native toxin; modeccin 6B largely precipitated and the electrophoretic separation was not attempted.

Two-dimensional electrophoresis of modeccin 4B and subunits. The pH 9.5 gels containing 20 μ g of either native or reduced modeccin 4B were subjected to electrophoresis in the second dimension in the presence of SDS. Two rows of spots appeared under the gel containing reduced modeccin: the first row, corresponding to the four bands with lower mobilities at pH 9.5, had the same molecular weight (mobility in the second dimension in the presence of SDS) as the larger subunit of modeccin. The second row, corresponding to the faster-migrating bands at pH 9.5, had the same molecular weight as the smaller subunit. The gel containing unreduced modeccin showed some heterogeneity in the second dimension in the presence of SDS, but all series of spots, aligned in the characteristic pattern described by O'Farrell (1975) for spots that were artifacts, were in that part of the gel containing higher-molecular-weight proteins.

Two-dimensional electrophoresis of the isoelectric-focusing gels of the isolated subunits of modeccin 4B confirmed that subunit A gives three and subunit B at least four spots with different charge and the same molecular weight.

Toxicity and effect on protein synthesis of modeccin 6B

The LD₅₀ of modeccin 6B was not determined, but it was observed that all rats given intraperitoneally 1 μ g of modeccin 6B/100g body wt. died, whereas all those receiving 0.1 μ g/100g body wt. survived. Thus the toxicity of modeccin 6B appeared intermediate between that of modeccin 4B and that of the same toxin reduced with 2-mercaptoethanol (Gasperi-Campani *et al.*, 1978).

Modeccin 6B was more potent than modeccin 4B in inhibiting protein synthesis in the reticulocyte-lysate system, with an ID₅₀ of 0.31 μ g/ml, comparable with that of reduced modeccin 4B (Gasperi-Campani *et al.*, 1978). The inhibitory activity of modeccin 6B was not enhanced by prior treatment of the toxin with 2-mercaptoethanol (see Table 2).

Fig. 3. Purification of the subunits of modeccin 4B by chromatography

Freeze-dried modeccin 4B (3.14 mg) was dissolved in 1.57 ml of 50 mM-Tris/HCl buffer, pH 8.5, containing 1% (v/v) 2-mercaptoethanol. After incubation at 37°C for 2 h the solution was applied to a column (12.5 cm $\times$ 0.7 cm) of Sepharose 4B, previously equilibrated with the Tris/mercaptoethanol solution. The column was washed with the same solution and was eluted with 0.2 M-galactose in Tris/mercaptoethanol (arrow). Fractions (1 ml) were collected at 10 ml/h.

Finally, modeccin 6B at concentrations up to 250 μ g/ml did not agglutinate pig erythrocytes, either in the presence or in the absence of neuraminidase.

Isolation and properties of the subunits of modeccin 4B

Isolation of the subunits of modeccin 4B was attempted on the assumption that they should have similar properties to the A and B subunits of ricin (Olsnes & Pihl, 1973a) and abrin (Olsnes & Pihl, 1973b). If so, the B subunit only should bind to Sepharose, thus allowing separation from the A subunit. Modeccin 4B was then incubated at 37°C for 2 h in phosphate-buffered saline, pH 7.2, in the presence of 1% 2-mercaptoethanol, and was loaded on a column of Sepharose 4B. No protein material emerged with the unretained effluent, but all remained attached to the column, from which it was eluted as a single peak with 0.2 M-galactose. However, when both the preincubation with 2-mercaptoethanol and the chromatography were performed at pH 8.5, part of the protein emerged as a single peak (peak A) with the unretained effluent, and part remained on the column and was eluted with 0.2 M-galactose (peak B) (Fig. 3). On SDS/polyacrylamide-gel electrophoresis the material of peak A corresponded to the subunit with mol.wt. 26 000 and the material of peak B corresponded to the 31 000-mol.wt. subunit (see Fig. 2). The 26 000-mol.wt. subunit had the characteristics of an A subunit, in that it

was a potent inhibitor of protein synthesis *in vitro*, with an ID_{50} of $0.066 \mu\text{g/ml}$, and did not agglutinate erythrocytes. The 31000-mol.wt. subunit had no effect on protein synthesis in the lysate system, at concentrations up to $100 \mu\text{g/ml}$, and agglutinated erythrocytes, thus behaving like a B subunit. The properties of the two forms of modeccin and of the A and B subunits of modeccin 4B are summarized in Table 2. Separation of the subunits of modeccin 6B was not attempted, since this toxin precipitated on reduction (see above).

Reactivity of modeccins and subunits with N-ethylmaleimide

Intact modeccins bind *N*-ethylmaleimide only in the presence of urea, as shown for modeccin 4B in Table 3 (lines 1 and 2 compared with line 3). To determine the number of disulphide bonds in the molecule, the free thiol groups (and all other groups reactive with *N*-ethylmaleimide) of modeccin were

irreversibly bound to non-radioactive *N*-ethylmaleimide in the presence of urea (line 3 of Table 3). Modeccin was then reduced with 2-mercaptoethanol: as shown in line 4, approximately three thiol groups per molecule became reactive with *N*-ethyl- $[^{14}\text{C}]$ maleimide. Since the reductive cleavage of disulphide bonds must give rise to an even number of half-cystine residues, the determinations of Table 3 are probably too low. When modeccin 4B treated with non-radioactive *N*-ethylmaleimide and labelled with *N*-ethyl $[^{14}\text{C}]$ maleimide in the thiol groups made available by reduction with 2-mercaptoethanol (as in line 4 of Table 3) was subjected to SDS/polyacrylamide-gel electrophoresis in the presence of 2-mercaptoethanol, approx. 75% of the total radioactivity was present in the slower-migrating band B and 25% in the faster-migrating band A. On the whole the results indicate that in the intact modeccin the two subunits are connected by a single disulphide bond, and that an additional intrachain disulphide bond is

Table 2. *Properties of modeccin 4B, of its A and B subunits, and of modeccin 6B*

Protein synthesis and haemagglutination were determined as described in the Experimental section. Modeccin was reduced by incubation at 37°C for 2 h in the presence of 1% 2-mercaptoethanol.

Preparation	Inhibition of protein synthesis (ID_{50} , $\mu\text{g/ml}$)	Haemagglutinating activity (lowest concentration, in $\mu\text{g/ml}$, giving visible agglutination)
Modeccin 4B		
Native	2.52	15.6
Reduced	0.1*	15.6
Subunit A	0.066	Inactive†
Subunit B	Inactive‡	3.9
Modeccin 6B		
Native	0.31	Inactive§
Reduced	0.38	Inactive§

* From Gasperi-Campani *et al.* (1978).

† At concentrations up to $125 \mu\text{g/ml}$.

‡ At concentrations up to $100 \mu\text{g/ml}$.

§ At concentrations up to $250 \mu\text{g/ml}$.

Table 3. *Thiol groups in modeccin 4B and in modeccin 4B reduced with 2-mercaptoethanol*

Determination of thiol groups was carried out as described in the Experimental section. Lines 2 and 3, modeccin (0.6 mg in 4 ml of phosphate-buffered saline) was preincubated for 2 h at 37°C with 20 mM non-radioactive *N*-ethylmaleimide in the absence (line 2) or in the presence (line 3) of 8 M-urea; excess *N*-ethylmaleimide was then removed by extensive dialysis against 50 mM-Tris/HCl buffer, pH 8.0. Line 4, modeccin as in line 3, but dialysed against Tris/HCl buffer, pH 8.5, was reduced for 2 h at 37°C with 1% 2-mercaptoethanol and then was extensively dialysed against 50 mM-Tris/HCl buffer, pH 8.0.

Pretreatment	<i>N</i> -Ethyl $[^{14}\text{C}]$ maleimide bound (pmol/pmol of protein)	
	Urea absent during assay	Urea present during assay
None	0.17	2.00
<i>N</i> -Ethylmaleimide	0.04	1.97
<i>N</i> -Ethylmaleimide, urea	0.05	0.10
<i>N</i> -Ethylmaleimide, urea, 2-mercaptoethanol	2.94	2.92

present in the B subunit. Similar results were obtained with modeccin 6B.

Both subunits of modeccin 4B, like the native toxin, reacted poorly with *N*-ethylmaleimide in the absence of urea; in the presence of urea the B subunit was only slightly more reactive than the A subunit. This result is explained by the observation that, when intact modeccin 4B after reaction with *N*-ethyl[¹⁴C]maleimide (as in line 1 of Table 3) was subjected to SDS/polyacrylamide-gel electrophoresis in the presence of 2-mercaptoethanol, 40% of the total radioactivity was present in the B band, and 60% in the A band. Clearly the reactive groups not engaged in disulphide bonds are more abundant in the A chain of modeccin 4B.

Attempts to separate on Sepharose 4B the A and B subunits labelled only in the cleaved disulphide bonds (obtained from modeccin reduced as in line 4 of Table 3) were unsuccessful, since both subunits were eluted together with the unretained effluent. Apparently the treatment with urea and/or *N*-ethylmaleimide affects the binding properties of the B subunit.

Discussion

Our results point to two main conclusions: (i) modeccin, like other toxins, consists of an A and a B subunit, of which the former inhibits protein synthesis and the latter binds to cells; (ii) several forms of modeccin can be obtained from the roots of *Adenia digitata*. These forms can be divided into two groups. One is modeccin 4B, the toxin originally isolated by affinity chromatography on Sepharose 4B (Stirpe *et al.*, 1978), and is characterized by a considerable charge heterogeneity, both of the whole molecule and of the constituent subunits. This heterogeneity was observed with the freeze-dried toxin as well as with the freshly prepared modeccin 4B. It is impossible to tell whether charge heterogeneity depends on the presence in the roots of natural variants of the toxin molecule, or is due to modifications occurring during the storage of the roots and/or the purification procedure. In any case, modeccin 4B as isolated can be considered as a mixture of isomeric proteins with mol.wt. 57000, differing from each other by small charge differences in one or both the constituent subunits.

The two subunits of modeccin 4B are joined together by a disulphide bridge and by non-covalent bond(s), since separation of the subunits after treatment with 2-mercaptoethanol occurs only in the presence of SDS or at alkaline pH. It should be noted that the subunits of ricin also are still kept together by weak forces after mild treatment with 2-mercaptoethanol (Lappi *et al.*, 1978).

The second form of modeccin, isolated on acid-treated Sepharose 6B (modeccin 6B), appears considerably more homogeneous on isoelectric focusing

and on electrophoresis at pH 9.5, and is more basic than modeccin 4B. Like the other form, modeccin 6B consists of two subunits held together by a disulphide bond. On treatment with 2-mercaptoethanol the solution of modeccin 6B showed a heavy precipitate. Thus separation of the subunits was observed only in the presence of SDS: hence the subunits were not isolated, and consequently it could not be ascertained which of them had the A and which the B function.

Modeccin 6B differs from modeccin 4B in that (i) it does not bind to Sepharose 4B, (ii) it does not agglutinate pig erythrocytes, (iii) it has a higher inhibitory effect on cell-free protein synthesis, and (iv) this effect is not enhanced by treatment with 2-mercaptoethanol.

Modeccin 6B binds to Sepharose only if the gel is subjected to mild acid hydrolysis. This treatment increases the adsorption capacity of Sepharose by splitting the galactan chains, thus exposing additional residues (Ersson *et al.*, 1973). This indicates that modeccin 6B has a weaker affinity than modeccin 4B for binding sites, which is consistent with the lack of haemagglutinating activity of the former lectin.

The smaller subunit of modeccin 6B is slightly larger (mol.wt. 27000) than the smaller subunit of modeccin 4B (mol.wt. 26000). The larger subunits of modeccin 4B and 6B show indistinguishable molecular weights on SDS/polyacrylamide-gel electrophoresis, and both contain an intrachain disulphide bond. In modeccin 4B the larger subunit is the B subunit, as indicated by its binding to Sepharose, by its haemagglutinating activity, and by the lack of effect on protein synthesis. Assuming that the larger subunit of modeccin 6B is also the B subunit, the different binding to Sepharose of modeccin 4B and 6B and the lack of haemagglutinating activity of modeccin 6B could be due to intrinsic differences among the respective B subunits, in spite of their identical molecular weights, or could be a consequence of the interaction of identical B subunits with different A subunits.

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