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The Exocellular DD-Carboxypeptidase–Endopeptidase from *Streptomyces albus* G

PURIFICATION AND CHEMICAL PROPERTIES

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The exocellular DD-carboxypeptidase–endopeptidase of *Streptomyces albus* G was purified to protein homogeneity and compared with the exocellular DD-carboxypeptidases–transpeptidases of *Streptomyces* R61 and *Actinomadura* R39. The *S. albus* G enzyme, as it is isolated, occurs in two forms. Enzyme I (30% of the total amount) and enzyme II (70% of the total amount) are identical in all respects, except that, by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate, enzyme I has an apparent mol.wt. (9000) that is half of that found by molecular-sieve filtration under non-denaturing conditions. Irrespective of the technique used, enzyme II has an apparent mol.wt. of about 18500.

Some actinomycetes excrete DD-carboxypeptidases during growth. Those excreted by *Streptomyces* R61 (the 'R61 enzyme') and by *Actinomadura* R39 (the 'R39 enzyme') were purified to protein homogeneity (Frère *et al.*, 1973, 1974). Both enzymes are able to catalyse concomitantly hydrolytic and transpeptidation reactions by transferring the X-D-alanyl- moiety of peptide donors X-D-alanyl-D-alanine either to water (DD-carboxypeptidase activity) or to suitable amino groups (transpeptidase activity); they are thus DD-carboxypeptidases–transpeptidases. These enzymes exhibit a high sensitivity to β-lactam antibiotics; with these antibiotics, they form rather stable, stoicheiometric complexes devoid of DD-carboxypeptidase and transpeptidase activities. The mechanisms involved in these various reactions have been studied extensively (Ghuysen, 1977*a*,*b*; Frère, 1977).

Streptomyces albus G excretes an exocellular DD-carboxypeptidase (the '*albus* G enzyme'; Ghuysen *et al.*, 1970; Leyh-Bouille *et al.*, 1970*a*), which, unlike the R39 and R61 enzymes, (1) requires very high concentrations of benzylpenicillin (10–50mM) to be inhibited (Leyh-Bouille *et al.*, 1970*b*), (2) is devoid of transpeptidase activity (Pollock *et al.*, 1972) and (3) hydrolyses with high efficiency a variety of *C*-terminal N^{α}-(D-Ala-D-) peptide bonds irrespective of the structure of the lateral chain of the terminal D-amino acid residue (Leyh-Bouille *et al.*, 1970*a*). As a consequence, the *albus* G enzyme is a lytic agent for those bacterial walls where such N^{α}-(D-Ala-D-) peptide linkages serve to cross-link the peptidoglycan subunits (Ghuysen *et al.*, 1970); it is thus a DD-carboxypeptidase–endopeptidase. The present paper describes its purification (in mg amounts) and some of its chemical properties.

Materials and Methods

Enzyme unit

One enzyme unit hydrolyses 1 μequiv. of D-Ala-D-Ala linkage/min and, at 37°C, from 6mM-Ac_2-L-Lys-D-Ala-D-Ala (i.e. a concentration equivalent to about 10 times the K_m value; Frère *et al.*, 1978), in 10mM-Tris/HCl buffer, pH 8.3, supplemented with 5mM-$MgCl_2$. The amount of free D-alanine released was measured enzymically (Frère *et al.*, 1976).

Protein determination

Proteins were measured (1) by the method of Lowry *et al.* (1951), (2) on the basis of A_{280} and A_{260} values (Kalckar, 1947) and (3) by measuring the amount of amino groups made available to dinitrophenylation by hydrolysis with 6M-HCl at 100°C for 20h (Frère *et al.*, 1976). During the various steps of the purification procedure, the proteins were as a routine determined by measuring the A_{280} of the solutions. For methods (1) and (3), bovine serum albumin was used as a standard.

Protein standards

The following proteins were used: bovine serum albumin (mol.wt. 68000); ovalbumin (mol.wt. 43000); carbonic anhydrase (mol.wt. 29000; chymotrypsinogen (mol.wt. 25700); myoglobin (mol.wt. 17000); lysozyme (mol.wt. 14500); cytochrome *c* (mol.wt. 12500); the spike parvalbumin III (mol.wt. 10000; Rao & Gerday, 1973).

Spectra

U.v. spectra were recorded with a Cary 17 double-beam spectrophotometer. Fluorescence emission was measured at 90° to the excitation beam (285nm) with an Aminco–Bowman recording spectrofluorimeter.

Polyacrylamide-gel electrophoresis

Electrophoreses at pH5.4 were performed at 4°C on cylindrical gels (0.7cm × 7cm) as described by Reisfeld *et al.* (1962). After a pre-run of 3h at 4mA/gel, the samples (40μg of protein) were submitted to electrophoresis for 150min at 4mA/gel. Equivalent gels were (i) stained with Coomassie Brilliant Blue R-250 for protein detection (scanning was performed at 265nm) or (ii) sliced into 2mm-thick discs. Each disc was eluted with 200μl of 10mM-Tris/HCl buffer (pH8.3)/5mM-$MgCl_2$ and the extracts were used for determination of enzyme activity.

Electrofocusing (pH range 3–10) of protein samples (40μg) was performed at 4°C on cylindrical gels (0.7cm × 7cm) as described by Wrigley (1969). Equivalent gels were (i) washed with a 2.5% (w/v) trichloroacetic acid solution for a total period of 6 days with two changes of the washing solution and then stained with Coomassie Brilliant Blue, (ii) sliced into 2mm-thick discs for determination of enzyme activity as described above, or (iii) sliced into 2mm-thick discs for pH measurements. For this purpose, each disc was eluted with 1ml of water and the pH values of the extracts were measured.

Electrophoreses in the presence of 0.1% sodium dodecyl sulphate of 20μg protein samples were performed at pH8.4 and 20°C on cylindrical gels (0.7cm × 9cm) as described by Laemmli & Favre (1973); the gels were stained with Coomassie Brilliant Blue.

Amino acid composition

Samples were hydrolysed and analysed as previously described (Frère *et al.*, 1974). Cysteine was measured after performic acid oxidation (Hirs, 1956) and tryptophan from the u.v. spectrum in alkali (Bencze & Schmid, 1957).

Peptide 'maps'

Protein samples (150μg in 500μl) were dialysed against 0.2M-$NaHCO_3$, boiled for 2min, cooled to 37°C, supplemented with 3μg of trypsin [Worthington, treated with L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone; a 1mg/ml solution in 1mM-HCl was used] and maintained for 8h at 37°C; fresh trypsin (3μg) was added after 2, 4 and 6h during the incubation period. The samples were freeze-dried, the pellets dissolved in 20μl of water and the solutions analysed by two-dimensional electrophoresis (at pH3.7) and chromatography (in acetic acid/butanol/water, 1:4:5, by vol.) on thin-layer cellulose plates by the technique of Gerday & Rao (1970). Staining was performed with ninhydrin.

Cell walls of Bacillus pasteurii

Cell walls of *B. pasteurii* in which the peptidoglycan interpeptide bonds are mediated through *C*-terminal N^{α}-(D-Ala-D-Asp) linkages (Ghuysen *et al.*, 1970) were used as substrate for lytic assays (see the introduction).

Growth of Streptomyces albus G

Streptomyces albus G was grown for 24h at 28°C with shaking in 1-litre flasks containing 500ml of peptone Oxoid medium (medium A described by Leyh-Bouille *et al.*, 1971). After two successive subcultures of increasing size, 100 litres of culture in exponential phase were used to inoculate 400 litres of the same medium contained in a 500-litre tank. This culture was grown at 28°C for 75h with mechanical stirring (120rev./min) and an air-flow rate of 100 litres/min at an air pressure of 1.5×10^5Pa. Silicone A emulsion (Dow Corning Co, Midland, MI, U.S.A.; 20ml) was used as antifoam. After centrifugation, the enzyme activity in the culture fluid was 4 munits/ml or 0.5munit/mg of protein.

Results

Purification of the enzyme

Step 1. The culture fluid (500 litres) was supplemented with 4kg wet wt. of Amberlite CG-50 (H^+ form), the pH of the suspension adjusted to 4.0 with acetic acid and the resin collected by centrifugation. The enzyme was quantitatively adsorbed on the ion-exchanger. All subsequent steps were carried out at 4°C. The enzyme was eluted from the Amberlite by treatment with 0.1M-Tris/HCl buffer, pH9.0. For this purpose, the resin was resuspended in the minimal volume of buffer (10 litres), the suspension [with the pH value adjusted to 8.0 with aq. conc. NH_3 (sp.gr. 0.880)] was poured into a column (15cm × 100cm) and washed with 0.1M-Tris/HCl buffer, pH9.0, until the eluate was devoid of enzymic

activity. The pooled active fractions (15 litres) were concentrated to 5 litres with a Millipore (Millipore Benelux S.A., Brussels, Belgium) Pellicon Cassette device (with PTGC 00005 membranes; mol.wt. cut-off 10000) and the concentrated solution was dialysed against 50 litres of 10mM-Tris/HCl buffer, pH8.3, containing 2mM-$MgCl_2$.

Step 2. After step 1, the enzyme solution was filtered through a column (6.5cm × 22cm) of DEAE-cellulose (MN 2100 DEAE, Macherey–Nagel, D-516 Düren, Germany) previously equilibrated against 10mM-Tris/HCl buffer, pH 8.3, containing 2mM-$MgCl_2$. Substantial amounts of pigments and inactive proteins were fixed on the column, whereas the enzyme was not retained. After washing with the same buffer until the effluent was free of enzyme activity, the active fractions were pooled (7 litres) and concentrated by ultrafiltration to 1 litre by using the Millipore Pellicon Cassette device.

Step 3. After step 2, the enzyme from the concentrated solution was adsorbed on a column (6.5cm × 20cm) of CM-cellulose (Cellex CM; Bio-Rad, Richmond, CA, U.S.A.) previously equilibrated against 10mM-Tris/HCl buffer (pH8.3)/2mM-$MgCl_2$. After washing until the filtrate contained less than 0.1mg of protein/ml, the enzyme was eluted from the column with a convex gradient of NaCl in the Tris/HCl/$MgCl_2$ buffer (mixing flask at constant volume: 800ml of buffer; added solution: 0.1M-NaCl in buffer). The enzyme was eluted at a NaCl concentration of about 0.09M. The active fractions were pooled (1 litre) and concentrated to 50ml by ultrafiltration through a Diaflo UM-10 membrane.

Step 4. After step 3, the enzyme solution was filtered through a column (4cm × 85cm) of Sephadex G-100 in 10mM-Tris/HCl, pH7.5, containing 2mM-$MgCl_2$ and 20mM-NaCl. The active fractions were pooled (200ml).

Step 5. After step 4, the enzyme was adsorbed on a column (3cm × 14cm) of CM-Sepharose (Pharmacia, Uppsala, Sweden) previously equilibrated against 40mM-Tris/HCl buffer (pH7.5)/2mM-$MgCl_2$/20mM-NaCl. After washing until the filtrate contained less than 0.1mg of protein/ml, the enzyme was eluted from the column with a convex gradient of NaCl [mixing flask at constant volume: 1 litre of 10mM-Tris/HCl (pH7.5)/2mM-$MgCl_2$/0.2M-NaCl; added solution: 10mM-Tris/HCl (pH7.5)/2mM-$MgCl_2$/0.4M-NaCl]. The enzyme was eluted at a NaCl concentration of about 0.25M. All the active fractions thus obtained had the same specific activity. They were pooled, concentrated by ultrafiltration with the help of a Diaflo UM-10 membrane (final concentration about 3mg of protein/ml) and dialysed against 10mM-Tris/HCl (pH8.3)/5mM-$MgCl_2$. The enzyme solution was stored in the frozen state at −20°C. Table 1 gives the total recoveries and enrichments in specific activity after each step of the purification procedure. In terms of enzyme activity, the final yield was 26%. Concentration of the enzyme by ultrafiltration at the end of steps 1, 2, 3 and 5 was not quantitative, about 10–20% of the activity occurring in the corresponding filtrates. Each filtrate was submitted to subsequent ultrafiltration, and, again, about 10–20% of the enzyme activity was not retained. In each case, the two concentrates thus obtained were combined and the solution was submitted to the ensuing step of the purification procedure.

Properties of the enzyme after step 5

(1) Filtration of 3mg of protein (i.e. 1ml of enzyme solution) through a column (1cm×60cm) of Sephadex G-75 in 10mM-Tris/HCl (pH8.3)/5mM-$MgCl_2$ and at 4°C yielded one single peak of enzymically active protein. All the fractions had the same specific activity. On the basis of the elution positions of standard proteins, the *albus* G enzyme had a mol.wt. of about 18500. (2) Polyacrylamide-gel electrophoresis at pH5.4 gave rise to one single band of

Table 1. *Purification of albus G* DD-*carboxypeptidase*

The value for total protein in step 5 was obtained from the A_{280} and A_{260} values (Kalckar, 1947). Values of 60 and 51mg were obtained by the method of Lowry *et al.* (1951) and by dinitrophenylation after hydrolysis respectively. Amino acid analysis agreed with the value of 64mg.

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg of protein)	Yield (%)	Volume (l)	Enrichment
Culture supernatant	4×10^6	2000	5×10^{-4}	100	500	1
Step 1	1.4×10^5	1900	1.4×10^{-2}	95	5	27
Step 2	4600	1433	0.31	72	1	600
Step 3	346	690	2.0	35	1	4000
Step 4	236	600	2.5	30	0.20	5000
Step 5	64	525	8.2	26	0.02	16400

Table 2. *Amino acid composition of the enzyme preparation after step 5 of the purification procedure*
The amount of enzyme hydrolysed was 90 μg; for details, see the text. Within the limits of experimental error, the amino acid compositions of enzyme I and enzyme II were identical with that of the enzyme preparation after step 5.

Amino acid	Amount of amino acid (nmol)	Weight (μg)	Residues/enzyme molecule (mol.wt. 19000)	Total mass (g/19000g)	% in number
Asx	97.4	11.2	20	2300	11.0
Thr	57.4	5.8	12	1212	6.6
Ser	60.0	5.2	12	1044	6.1
Glx	66.5	8.6	14	1806	7.7
Pro	32.2	3.1	6	582	3.3
Gly	142	8.1	30	1710	16.5
Ala	133	9.5	28	1988	15.4
Val	37.2	3.7	8	792	4.4
Met	18.2	2.4	4	524	2.2
Ile	26.4	3.0	6	678	3.3
Leu	40.6	4.6	8	904	4.4
Tyr	17.4	2.8	4	652	2.2
Phe	27.0	4.0	6	882	3.3
His	21.3	2.9	4	548	2.2
Lys	21.9	2.8	4	512	2.2
Arg	49.0	7.6	10	1550	5.5
½-CyS	11.0	1.1	2	408	1.1
Trp	21.2	4.0	4	748	2.2
Total		90.4	182	18840	99.6

protein and the enzyme activity was found at the same position in an equivalent gel. The migration was 0.7cm towards the cathode (for experimental conditions, see the Materials and Methods section). (3) In turn, gel electrofocusing gave rise to a main band of protein (94% of the total amount) that focused at pH8.5; the enzyme activity was also found at the same position in an equivalent gel. Some protein impurity (6%) focused at a lower pH value. After both gel electrophoresis at pH5.4 and gel electrofocusing, the enzyme recovery was low (about 10%); thus enzyme denaturation occurred during the process and was probably due to the lack of Mg^{2+} in the gels. (4) The sum of the amino acid residues found after acid hydrolysis (Table 2) corresponded to the nominal amount of protein hydrolysed. (5) Finally, and surprisingly, polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate revealed the presence of two proteins. The migration of the main protein (70% of the total amount) agreed with the expected mol.wt. of about 18500, whereas that of the minor protein (30%) indicated a mol.wt. of about 9000. On the basis of these determinations, the enzyme with mol.wt. 18500 was referred to as enzyme II and that with mol.wt. of 9000 as enzyme I.

Isolation of enzymes I and II

A large sample (45mg) of the enzyme preparation obtained after step 5 was filtered at 4°C through a column (3cm×45cm) of Sephadex G-50 (superfine grade) in 10mM-Tris/HCl (pH8.3)/5mM-$MgCl_2$. As before, one single peak of enzymically active protein was obtained; the K_D value indicated a mol.wt. of about 18500 and all the fractions exhibited the same specific activity (Fig. 1). However, analysis of the individual fractions by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate revealed that fractions 15–18 contained almost exlusively enzyme II, whereas fractions 19–22 contained almost exclusively enzyme I (Figs. 1 and 2). The pooled fractions 17–18 and the pooled fractions 19–22 were used to further characterize enzyme II and enzyme I respectively.

Properties of enzymes I and II

Within the limits of experimental error: (1) enzymes I and II had the same amino acid composition as that of the enzyme mixture obtained after step 5 of the purification procedure (Table 2); (2) both enzymes gave rise to identical peptide 'maps' (Fig. 3); (3) they showed identical absorption and fluorescence-emission spectra (Fig. 4); the $A^{1\%}_{1cm}$ at 280nm was 10.0 for both of them; (4) they exhibited the same specific activity on Ac_2-L-Lys-D-Ala-D-Ala (8 μequiv. of D-Ala-D-Ala hydrolysed/min per mg of protein under conditions of enzyme saturation); (5) they hydrolysed the *B. pasteurii* cell walls at approximately the same rate. Thus a suspension of 1.5mg of cell walls/ml in 10mM-Tris/HCl (pH8.5)/5mM-$MgCl_2$ had its absorption value at 650nm decreased by 50% after incubation in the presence of 30 μg of protein for

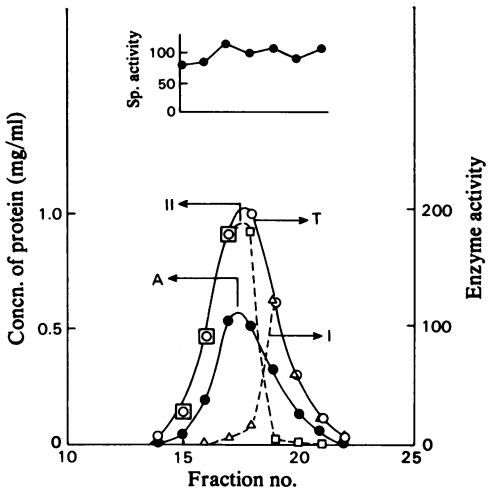

Fig. 1. *Gel filtration of the enzyme preparation after step* 5 *of the purification procedure* (45 *mg*) *on Sephadex G-50* (*superfine grade*) *in* 10 *mM-Tris/HCl* (*pH* 8.3)/4 *mM-*$MgCl_2$ *and at* 4°*C*

Fractions (12ml) were collected; for other details see the text. Total protein concentrations (curve T, ○) (in mg/ml) were determined by u.v.-absorbance measurements. Enzyme activity (curve A, ●) (in nmol of D-alanine liberated/μl of each fraction) was measured by incubating samples (0.2–1.0μl) of each fraction for 15min at 37°C with 1.7mM-Ac_2-L-Lys-D-Ala-D-Ala in a total volume of 30μl of 10mM-Tris/HCl (pH8.3)/5mM-$MgCl_2$. The insert shows the ratios of enzyme activity/protein concentration (in nmol of D-alanine/μg of protein). Enzyme I (curve I, △) and enzyme II (curve II, □) (in mg of protein/ml) were measured by submitting a sample of each fraction to polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate and scanning the stained gels at 265 nm.

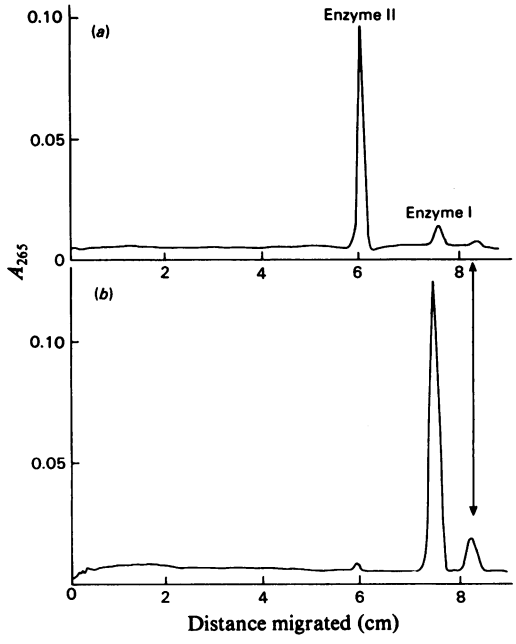

Fig. 2. *Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate of fractions* 18 (*a*) *and* 19 (*b*) *of Fig.* 1

After staining with Coomassie Brilliant Blue, the gels were scanned at 265nm (a 20μl sample of fraction 18 and a 50μl sample of fraction 19 were used). Enzyme I had mol.wt. 9000 and enzyme II, mol.wt. 18500. Arrows indicate the position of Bromophenol Blue.

20min at 37°C (total volume 325μl). Note that in 5mM-sodium phosphate buffer, pH7.0, the R61 enzyme (100μg of protein/ml, final concentration) had no lytic effect on the cell-wall suspension even after 6h of incubation at 37°C; (6) they were unable to catalyse transfer reactions with the substrate system 2mM-Ac_2-L-Lys-D-Ala-D-Ala + 30mM-D-[^{14}C]alanine (Pollock *et al.*, 1972); (7) they were not inhibited by the anti-(R61 enzyme)antiserum (Nguyen-Distèche *et al.*, 1977); (8) they exhibited the same low sensitivity to cephalosporin C. Thus, for example, when enzyme (0.05μg of protein) and Ac_2-L-Lys-D-Ala-D-Ala (2mM) were incubated together for 15min at 37°C in 30μl of 10mM-Tris/HCl, pH8.0, supplemented with 5mM-$MgCl_2$, both enzymes were inhibited by 22 and 37% in the presence of 0.27mM- and 0.54mM-cephalosporin C respectively; (9) when filtered separately on the same column of Sephadex G-50 (superfine grade) as above (4°C), each enzyme gave rise to one single peak of protein with a mean K_D value indicating a mol.wt. of about 18500. As shown by polyacrylamide-gel electrophoresis in sodium dodecyl sulphate, all the fractions originating from enzyme II contained enzyme II (mol.wt. 18500), whereas most of the fractions originating from enzyme I contained enzyme I (mol.wt. 9000). From the foregoing it was concluded that, to all appearances, enzyme I and enzyme II were identical, except for the effect caused by sodium dodecyl sulphate on enzyme I. By electrophoresis in the presence of this denaturing agent, enzyme I had a molecular weight half that found by molecular-sieve filtration under non-denaturing conditions.

Assays of interconversion between enzyme I and enzyme II

Performic acid oxidation (Hirs, 1956), reduction by dithiothreitol followed by alkylation with iodoacetate (Crestfield *et al.*, 1963), and prolonged incubations (up to 100h) at 37°C and at 60°C in the presence of 1%

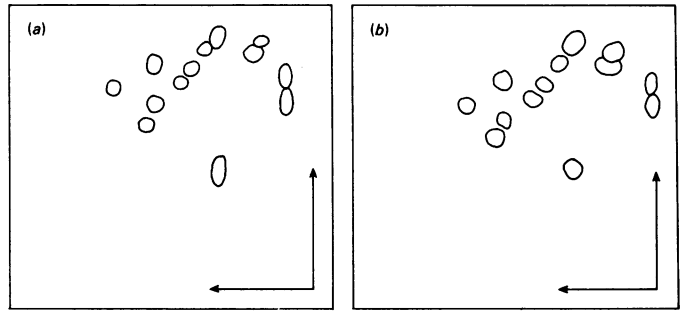

Fig. 3. *Peptide 'maps' of enzyme I (a) and enzyme II (b)*
In each case, protein (150 μg) was digested with trypsin. The arrows indicate the directions of ascending chromatography and electrophoresis.

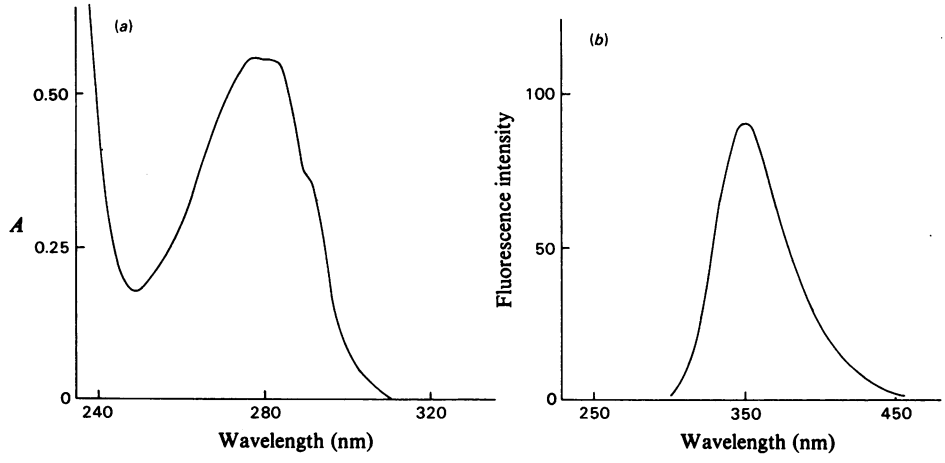

Fig. 4. *Absorption (a) and fluorescence-emission (b) spectra of enzyme II*
The spectra were determined on solutions containing either 0.04 mg of protein/ml (fluorescence) or 0.57 mg of protein/ml (absorption). Enzyme I showed superimposable spectra. The solutions were in 10 mM-Tris/HCl buffer, pH 7.7, containing 4 mM-$MgCl_2$. For the fluorescence spectrum, excitation was at 285 nm. Fluorescence intensity is in arbitrary units.

sodium dodecyl sulphate either as such or supplemented with mercaptoethanol (up to 5%), failed to transform enzyme II into enzyme I. Free thiol groups could not be detected in enzyme I with 5,5'-dithiobis-(2-nitrobenzoic acid) in the presence of 5 M-urea. However, after treatment with saturating concentrations of urea for 24h at 37°C, enzyme I was partially transformed into enzyme II. On the contrary, when incubated under the same conditions, but in the presence of 1% mercaptoethanol, enzyme II was not transformed into enzyme I. Exposure to high temperatures (60–100°C), to high (12.0) and low (4.0) pH values, did not cause any detectable interconversion. Occasionally, partial interconversion was observed, but the observations were not reproducible. Thus, for example, when purified enzyme I was submitted to subsequent filtration on Sephadex G-50 (see above), those fractions collected at the front of the peak contained enzyme II in amounts disproportionate with that present in the original preparation. Similarly, storage of enzyme II in the frozen state at −20°C sometimes resulted in the formation of small amounts of enzyme I. The significance of these observations is dubious. Clearly, however, there is no rapid equilibrium between enzyme I and enzyme II.

Discussion

The DD-carboxypeptidase excreted by *Streptomyces albus* G and as it was isolated occurs in two forms: enzyme II and enzyme I. Enzyme II largely predominates in the final preparation (70% of the total activity). On the basis of both the K_D value observed by molecular-sieve filtration under non-denaturing con-

ditions, and the migration observed by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate, enzyme II has a mol.wt. of about 18500. This molecular weight is considerably lower than those of the exocellular R61 enzyme (mol.wt. 38000; Frère *et al.*, 1973) and the exocellular R39 enzyme (mol.wt. 53000; Frère *et al.*, 1974) (see the introduction). The three enzymes, however, present some similarities: (1) altogether, the seven residues aspartic acid+asparagine, glutamic acid+glutamine, glycine, alanine, valine, leucine and threonine, found in the R39, R61 and *albus* G enzymes represent 74, 67 and 63% respectively of the total number of amino acid residues; (2) each enzyme contains very few sulphur-containing residues (3.3% for the *albus* G enzyme); (3) the proportions of polar residues are 41% for the *albus* G enzyme, 40% for the R39 enzyme and 47% for the R61 enzyme; (4) in all cases, the glutamic acid+glutamine and aspartic acid+asparagine residues greatly outnumber the basic ones. This latter property well explains the negatively charged character of the R61 and R39 proteins. In contrast, the *albus* G enzyme has a relatively high isoelectric point (pH 8.5) and therefore most of its acidic residues must be amidated. Maximum fluorescence emission is at 318–320nm with the R61 enzyme (Nieto *et al.*, 1973), at 339–340nm with the R39 enzyme (Frère *et al.*, 1974) and at 350nm with the *albus* G enzyme; this latter value is very close to that with free tryptophan (347nm). With Ac_2-L-Lys-D-Ala-D-Ala as substrate, turnover numbers of the R39 and R61 enzymes are high (3300 and 1050 respectively; Frère *et al.*, 1973, 1974), whereas that of the *albus* G enzyme is low (150 for a catalytic unit with a mol.wt. of 18500). Out of the three exocellular DD-carboxypeptidases studied, the *albus* G enzyme is the only one that is unable to catalyse transpeptidation reactions (Pollock *et al.*, 1977) and the only one that is able to hydrolyse with high efficiency a variety of *C*-terminal N^{α}-(D-Ala-D-) linkages that cross-link the peptidoglycan units in certain bacterial walls and, consequently, to cause cell-wall lysis (Ghuysen *et al.*, 1970). The peptidoglycan cross-linking enzyme system in all bacteria exhibits various DD-carboxypeptidase, transpeptidase and endopeptidase (i.e. lytic) activities (Ghuysen, 1977*c*). Altogether the R39, R61 and *albus* G enzymes are models representing the whole range of reactions catalysed.

The *albus* G enzyme I is identical with enzyme II in all respects, except that (1) when a mixture of enzyme I and enzyme II was filtered on Sephadex under non-denaturing conditions, enzyme I was eluted immediately after enzyme II and (2) by polyacrylamide-gel electrophoresis in sodium dodecyl sulphate, enzyme I has a mol.wt. (9000) that is half the value found by Sephadex filtration. The simplest hypotheses that might explain these observations are that (1) both enzymes I and II would consist of two polypeptide chains of similar size, (2) the two polypeptide chains would be linked together by at least one covalent bond in enzyme II and would be held together by more fragile bonds in enzyme I (with the result that dissociation into monomeric units could occur only in the case of enzyme I), and (3) under non-denaturing conditions, both enzymes would have at least very similar conformations, therefore exhibiting identical enzymic and chemical properties. The occurrence of a disulphide bond in enzyme II is not impossible (although this bond, if present, should have very peculiar properties). In fact, the nature of the forces that hold together the two postulated polypeptides in enzymes I and II, the form under which the enzyme is excreted during growth of the *Streptomyces*, whether the catalytic unit is the monomeric polypeptide or the dimeric protein, and whether or not enzyme I is produced by a limited proteolysis of enzyme II, are questions to which at present, answers cannot be given.

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