Protein and Carbohydrate Moieties of a Preparation of β -Lactamase II

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1. A crystalline preparation of β -lactamase II has been separated into two moieties by gel filtration on a column of Sephadex G-100. 2. The first moiety consisted mainly of carbohydrate and showed virtually no β -lactamase activity. 3. The second moiety was a protein of molecular weight 22500, which was enzymically active. 4. The protein moiety, like the original protein-carbohydrate complex, required Zn^{2+} for β -lactamase activity. It did not differ significantly from the complex in its behaviour to a number of cephalosporin substrates, but was less stable to heat than the complex. 5. About 30% of the total β -lactamase activity was lost when the protein-carbohydrate complex was separated into the two moieties. This activity was regained when the protein and carbohydrate moieties were mixed, but the mixture did not show the heat stability of the original complex.

Although β -lactamase I and β -lactamase II are both produced by *Bacillus cereus* 569H the latter enzyme differs from the former in requiring Zn^{2+} as a cofactor (Sabath & Abraham, 1966), in showing a wider range of activity, and in being less readily inactivated by heat (Crompton, Jago, Crawford, Newton & Abraham, 1962; Kuwabara & Abraham, 1967). Crystalline β -lactamase II prepared by Kuwabara (1970) had a higher molecular weight than crystalline β -lactamase I (Lloyd & Peacocke, 1970) and unlike the latter, which gave only amino acids on acid hydrolysis, yielded substantial quantities of neutral sugars and amino sugars in addition to amino acids (Kuwabara, Adams & Abraham, 1970). However, the nature of the association of carbohydrate with protein in this preparation of β -lactamase II and the extent to which the carbohydrate moiety was responsible for its characteristic β -lactamase activity remained unknown.

The present paper describes the separation of the carbohydrate moiety from the protein moiety of crystalline β -lactamase II and some of the properties of the two components.

MATERIALS AND METHODS

Enzyme preparations. Two crystalline preparations (*a* and *b*) of β -lactamase II from *B. cereus* 569H were used. These preparations were obtained as described by

Kuwabara (1970), the purification process being taken to stage 8. They had a specific activity of 2.59×10 units/mg⁶ of protein N with cephalosporin C as substrate. The Zn^{2+} -containing buffer BZ and the substrates used were also as described by Kuwabara (1970).

Assay of β -lactamase activity. The unit of activity is the amount of enzyme required to hydrolyse 1 μ mol of substrate in 60 min at pH 7.0 (Pollock & Torriani, 1953). Assays were carried out with a pH-stat (type SBR2/SBU 1 titrator) in connexion with type TT1 automatic titrator and with cephalosporin C as substrate as described by Kuwabara (1970).

Determination of protein. The concentration of protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) except in the case of samples used for determinations of molecular weight and for amino acid analysis. The concentration of protein in the latter samples was determined by comparing the refractive index of a solution with that of its diffusate in a Brice-Phoenix differential refractometer and assuming a specific refractive increment of 0.183 ml/g.

Determination of molecular weights. The molecular weights of the two moieties of β -lactamase II were determined by sedimentation equilibrium by using the procedures described by Lloyd & Peacocke (1970). The speeds used were 12590 rev./min for the carbohydrate moiety and 15220 rev./min for the protein moiety, and in each case the temperature was 20°C. The partial specific volume of the protein was measured by comparing the density of a solution with that of its diffusate by means of an Anton-Paar digital densimeter. That of the carbohydrate moiety was calculated from its composition as described by Lloyd & Peacocke (1970).

Determination of protein and carbohydrate by weight. For this purpose samples in buffer BZ containing about 2mg of carbohydrate or protein in 0.5ml, were desalted by gel filtration on a column of Sephadex G-25 (45 cm $\times$ 0.3 cm diam.), the resulting solution in water was freeze-dried, and the residues were weighed.

Amino acid analysis. Amino acids in acid hydrolysates of the β -lactamases (200 μ g) were determined as described by Moore, Spackman & Stein (1958) and Spackman, Stein & Moore (1958) with an amino acid analyser from the Locarte Co. The protein was hydrolysed with 6M-HCl at 110°C for 17h. Cyst(e)ine was determined as cysteic acid as described by Moore (1963). The losses of serine and threonine on hydrolysis were assumed to be 10% and 5% respectively. Tryptophan was determined after alkaline hydrolysis of the enzymes as described by Noltmann, Mahowald & Kuby (1962). The tyrosine/tryptophan ratio was determined spectroscopically by the method of Holiday (1936).

Carbohydrate analysis. This was kindly carried out by Dr P. W. Kent and Mr P. F. Daniel by g.l.c. of the trimethylsilyl ethers of the methyl glycosides, according to the method of Sweeley, Bentley, Makita & Wells (1963), as modified by Clamp, Dawson & Hough (1967).

A freeze-dried sample of the carbohydrate preparation was hydrolysed in duplicate (approx. 1.5mg+50 μ g of mannitol as internal standard) in methanolic 1M-HCl for 24h at 80°C. The samples were then neutralized with Ag_2CO_3 and reacylated in 10% (v/v) acetic anhydride. They were then evaporated to dryness on a rotary evaporator and finally dried *in vacuo* over phosphorus pentoxide. The samples were silylated in 100 μ l of a mixture of pyridine-hexamethyldisilazane-trimethylchlorosilane (5:1:1, by vol.) for 10min, and then 1 μ l was

injected on to a Pye series 104 model 24 chromatograph (W. G. Pye & Co. Ltd., Cambridge, U.K.) with dual flame-ionization detectors, temperature-programmed from 140°C to 210°C at 1°/min. The columns were packed with 3% SE-30 on Diatoport-S 80-100 mesh (from Hewlett-Packard Ltd., Slough, Bucks., U.K.). Carrier gas was argon, flow rate 40ml/min.

RESULTS

Separation of the carbohydrate and protein moieties of β -lactamase II. The crystalline β -lactamase II employed in these experiments had been dissolved for storage in buffer BZ at a concentration of about 15mg/ml and the solution adjusted to 65% saturation with ammonium sulphate, the pH of which had been adjusted to 7.0 with ammonia. The suspensions had been kept under these conditions at 4°C for about 3 months without loss of activity. The enzyme was brought into solution by removing the ammonium sulphate by dialysis against 10mM buffer BZ at 4°C. A solution of the enzyme (9.48mg in 4.5ml of buffer BZ) was added to a column (150cm $\times$ 1.0cm diam.) of Sephadex G-100 at room temperature. Elution was carried out with 10mM buffer BZ at a flow rate of 0.18ml/min and fractions were collected every 5min. The values of E at 253nm for these fractions indicated that the enzyme preparation had been separated into two major fractions and one minor fraction (Fig. 1). Moiety I (fractions 21-32) was virtually

Fig. 1. Gel filtration of a preparation of crystalline β -lactamase II on a column (150cm $\times$ 1.0cm diam.) of Sephadex G-100. ----, E_{253} ; $\circ$, β -lactamase II activity. For experimental details see the text.

inactive, but moiety II (fractions 33–35) showed the enzymic activity of β -lactamase II. Although most (94%) of the weight of the original enzyme was recovered in the two moieties, only about 60% of the original enzymic activity was found in moiety II. The specific activity of freshly prepared moiety II (2.31×10^6 units/mg of protein N) was 35% lower than that of the original enzyme preparation. Detail of recoveries of weight and activity are given in Table 1.

To remove contamination of moiety II with moiety I due to overlap in the elution, a sample (4ml) of the combined fractions 33–55 (containing 3.2mg) was added to a second column (50cm $\times$ 1.0 cm diam.) of Sephadex G-100. Elution was carried out with 10mM buffer BZ, pH 7.0, and fractions (0.4ml) were collected every 5min. The recovery of protein in fractions 22–33 was 2.8mg. The resulting preparation of moiety II, which was free from moiety I, did not differ significantly in specific activity from the preparation obtained from the first column.

A sample of moiety I (1.0ml; 1.91mg) was desalted on a column (45cm $\times$ 0.5cm diam.) of Sephadex G-25 and fractions (1ml) collected every 5min. The preparation obtained from fractions 7–15 was used for amino acid analysis.

Molecular-weight determinations. The molecular weight of moiety II (protein moiety) was determined at two concentrations, 2.6mg/ml and 1.3mg/ml, and the values obtained were 22500 and 22800 respectively. These values are probably not significantly different and the molecular weight of this moiety may be taken as 22500. The results indicated the presence of a small amount of component of much higher molecular weight very similar to that found with intact β -lactamase II (Lloyd & Peacocke, 1970).

Sedimentation-equilibrium experiments were done with moiety I (carbohydrate moiety) at three concentrations (8.2, 4.1 and 2.0mg/ml). The plot of $\ln c$ against r^2 (where c is the concentration of the solution at a distance r from the centre of rotation) for the most concentrated solution was concave towards the r^2 axis; the same plot was slightly sigmoid in shape for the solution of intermediate

concentration and convex to the r^2 axis for the most dilute solution. These results indicate that the moiety was aggregating and that its solution was thermodynamically non-ideal. The minimum apparent molecular weight observed was 19000 and the maximum 56000 (both in the solution of lowest concentration). The concentration ranges of the three solution columns overlapped sufficiently to show that the apparent molecular weight was a unique function of the concentration of the solution, indicating a truly reversible aggregation. However, since the molecular weight of intact β -lactamase II is 35600 (Lloyd & Peacocke, 1970) and that of the protein moiety II is 22500 the monomeric form of the carbohydrate moiety I should not have a molecular weight greater than 13000. The results of these experiments indicate that this monomer aggregates very readily, at least as far as the tetrameric form.

Composition of carbohydrate moiety I. The amino sugars of the original preparation of β -lactamase II were found exclusively in moiety I. The values for amino acids, glucosamine and galactosamine obtained with the amino acid analyser from two different preparations are given in Table 2, the number of residues being related to a weight of moiety I of 13000g. Although most of the amino acids of the original preparation were detected in this moiety none was present in an amount sufficient to correspond to one residue/13000g.

The sugars identified by g.l.c. were *N*-acetylglucosamine (17), *N*-acetylgalactosamine (10), glucose (19) and galactose (1). The values in parentheses give the number of residues found relative to a value of 10 for *N*-acetylgalactosamine, the latter value being the mean of those obtained for galactosamine from the amino acid analyser. Relative values are given because the sample analysed contained buffer. In addition to these sugars a peak was observed just before the mannitol standard (relative retention time 0.97) which was not identified. The area under this peak was about 50% of that under galactosamine. The relative retention time was close to that of *N*-acetyl- β -mannosamine but this assignment was not confirmed since no peak was observed with relative retention time 1.03 corresponding to the α -anomer.

Table 1. Amounts and activities of the carbohydrate and protein moieties obtained from crystalline β -lactamase II

For details of the preparations see the text.

Substance	Total activity (units)	Weight (mg)	Units recovered (%)	Weight recovered (%)	Specific activity (units/mg of protein N)
β -Lactamase II (protein-carbohydrate complex)	3.89×10^6	9.48	100	100	3.53×10^6
Carbohydrate moiety	2.78×10^3	2.48	0.07	26	—
Protein moiety	2.36×10^6	6.48	60.6	68 } (94)	2.31×10^6

Table 2. *Composition of the carbohydrate and protein moieties of crystalline β -lactamase II*

The preparation of protein and carbohydrate moieties from crystalline β -lactamase II were (a) those used for the determination of molecular weights and (b) a similar preparation from a different batch of starting material. Hydrolysis and amino acid analysis were carried out as described in the Materials and Methods section. Values in column (c) are calculated from the mean of those given for crystalline β -lactamase II (protein-carbohydrate complex) by Kuwabara *et al.* (1970) based on an arbitrary value of three residues for histidine. The values in column (d) are calculated from the mean of those given for β -lactamase I by Kuwabara *et al.* (1970) but are related to 22500 g of protein for comparison with the protein moiety of β -lactamase II. No correction was made for destruction of amino sugars on hydrolysis. Values represent the number of residues found in the stated molecular weight. The values for tryptophan in the protein moiety of β -lactamase II were determined by spectroscopic estimation of the tyrosine/tryptophan ratio.

Amino acid or amino sugar	β -Lactamase II					
	Carbohydrate moiety (13000 g)		Protein moiety (22500 g)		Protein- carbohydrate complex (relative)	β -Lactamase I (22500 g)
	(a)	(b)	(a)	(b)	(c)	(d)
Lys	0.1	0.4	16	13	17	17
His	0.0	0.1	4	4	3	3
Arg	0.0	0.1	4	6	8	13
Cys*			1	1	0.8	0.3
Asp	0.1	0.7	23	22	26	25
Thr	0.4	0.4	14	15	14	17
Ser	0.4	0.4	11	13	13	12
Glu	0.1	0.5	18	9	20	18
Pro			8	10	9	9
Gly	0.4	0.4	18	18	18	16
Ala	0.2	0.3	15	15	18	23
Val	0.5	0.5	15	15	15	10
Met	0.0	0.1	2	2	2	3
Ile	0.6	0.3	17	13	16	15
Tyr	0.1	0.6	5	10	8	7
Phe	0.6	0.5	4	8	6	6
Try	0.0	0.1	2	3	2	3
Glucosamine	18	13				
Galactosamine	8	12				

* Determined as cysteic acid.

The u.v.-absorption spectrum of moiety I showed a broad band between 250 nm and 300 nm with a shallow maximum ($E_{1\text{ cm}}^{1\%}$ 3.0) at 280 nm, and also a low plateau ($E_{1\text{ cm}}^{1\%}$ 1) extending to 240 nm.

The molecular weight of moiety I determined from its composition is only about 9000 compared with the difference of 13000 between the intact enzyme and its protein moiety. However, the value of 9000 is subject to considerable error in view of the variations in the values for hexosamines obtained in the amino acid analyser after hydrolysis under vigorous conditions. Further, the third and minor component of the eluate from the Sephadex G-100 may contribute to the value of 13000.

Composition of the protein moiety II. The results of amino acid analysis of moiety II are given in Table 2, the number of residues being calculated in relation to a weight of protein of 22500 g.

The amino acid composition of the protein moiety was similar to that given by Kuwabara *et al.* (1970)

for crystalline β -lactamase II. Cysteine was apparently present in the latter and amino acid analysis of a hydrolysate of the protein moiety that had been oxidized by performic acid indicated the presence of cysteic acid in an amount corresponding to one residue/molecule of protein.

The u.v.-absorption spectrum of the protein moiety at pH 7.0 showed a maximum at 277 nm ($E_{1\text{ cm}}^{1\%}$ 8.7) compared with one at 280 nm ($E_{1\text{ cm}}^{1\%}$ 8.2) for the original preparation of β -lactamase II.

Enzymic properties of the protein moiety. The β -lactamase activity of the protein moiety, like that of the original crystalline β -lactamase II, was Zn^{2+} -dependent. Values obtained for V_{max} (relative to that for benzylpenicillin) and K_m with cephalosporin C, cephalothin, methicillin, oxacillin and cloxacillin as substrates, did not differ significantly from the corresponding values obtained with the original preparation (Kuwabara & Abraham, 1967).

When the protein moiety was kept for about 3 months at 4°C in 65% saturated ammonium sulphate, pH 7.0, it lost about 90% of its enzymic activity although the original β -lactamase II was stable under these conditions. Gel filtration of the stored and largely inactive material on Sephadex G-100 resulted in the appearance of several inactive fragments in the effluent after the appearance of the small proportion of active protein moiety which still remained.

When a solution of the protein moiety (18h after preparation) was heated at 65°C in buffer BZ, pH 7.0, its activity against cephalosporin C and benzylpenicillin fell nearly to zero within 30min (Fig. 2). In this it resembled β -lactamase I, but contrasted with the original β -lactamase II which retained about 90% of its activity under similar conditions (Kuwabara, 1970). When the carbohydrate moiety was mixed with the protein moiety the thermostability of the original complex was not restored.

Effect of the carbohydrate moiety on the enzymic activity of the protein moiety. When the β -lactamase activity of the protein moiety was measured in the presence of increasing amounts of the carbohydrate moiety the activity was found to vary with the carbohydrate moiety/protein moiety weight ratio. With cephalosporin C as a substrate the activity increased by 18% when the ratio was 0.13 and the increase reached a maximum (50% greater than that of the protein moiety alone) when the ratio was raised to 0.26. When the ratio exceeded this value the activity declined, apparently in three stages (Fig. 3). With a ratio of more than 2.0 the carbohydrate moiety inhibited the activity of the protein and when the ratio reached 3.9 the activity of the latter fell to less than 5% of its original value.

The specific activity of the protein moiety

(2.31×10^6 units/mg of protein N) was lower than that of native β -lactamase II (3.53×10^6 units/mg of protein N). When the carbohydrate moiety was added to the protein moiety to give a ratio of 0.260 the apparent specific activity of the latter rose to 3.48×10^6 units/mg of protein N.

DISCUSSION

The carbohydrate and protein moieties of the original crystalline β -lactamase II were not separated by precipitation with ammonium sulphate, by chromatography on DEAE-cellulose, or by the heat treatment which inactivated β -lactamase I (Kuwabara, 1970). However, the loss of β -lactamase II activity when β -lactamase I was purified by adsorption on powdered glass or Celite (Sabath & Abraham, 1966; Kuwabara, 1970) raised the question whether a protein-carbohydrate linkage was effected by those procedures.

The earlier sedimentation-equilibrium studies on β -lactamase II (Lloyd & Peacocke, 1970) gave no indication of any tendency of this enzyme preparation to separate into two moieties. Moreover, the fact, reported here, that the activity of the two moieties together is higher than that of the protein moiety alone (even though the carbohydrate moiety itself has no enzymic activity) indicates that the original protein-carbohydrate complex does not dissociate extensively into its two moieties on dilution. Hence, the separation of the complex

Fig. 2. Stabilities of the original β -lactamase II (●) and its protein moiety (○) respectively at 65°C in buffer BZ (for details see the Materials and Methods section).

Fig. 3. Effect of the carbohydrate moiety on the enzyme activity of the protein moiety of β -lactamase II. Different amounts of the carbohydrate moiety were added to a standard amount of the protein moiety in the solution used for assay and the mixture was kept at 30°C for 3 min before addition of cephalosporin C as substrate (for details see the Materials and Methods section).

into two moieties by passage through a column of Sephadex G-100 was unexpected. The appearance of the carbohydrate moiety before the protein moiety in the percolate from the column can be attributed to the ability of the carbohydrate to aggregate readily and this aggregation is presumably responsible, at least in part, for the separation observed. However, the possibility that dissociation of the complex was facilitated by an interaction with the Sephadex, or that a change in the original enzyme preparation had occurred on storage through microbial contamination, has not been eliminated.

Analysis of the carbohydrate moiety showed that it contained glucosamine and galactosamine in relative amounts which were similar to those in the original enzyme (Kuwabara *et al.* 1970), but the amount of glucose relative to the amount of amino sugar was somewhat higher in the carbohydrate moiety than in the original enzyme and the amount of galactose was much lower. Mannose and fucose, which were found in the original enzyme, were not detected in the carbohydrate moiety. Whether any of the missing neutral sugars have remained attached to the protein moiety, or are present in the third and minor component of the eluate from Sephadex G-100, remains to be determined.

The molecular-weight measurements on the carbohydrate moiety showed clearly that it aggregated very readily. This conclusion is supported by the fact that it emerged from the Sephadex G-100 column close to the void volume of the column. The apparent u.v. absorption of this moiety can probably be attributed to slight turbidity resulting from this aggregation together with a small contribution from the amino acids found in this fraction.

The finding that the protein moiety lacked the relative thermostability of the original β -lactamase II would be consistent with the view that the carbohydrate moiety played a role in this thermostability, as carbohydrate appears to do in the case of a number of glycoproteins and carbohydrate-protein complexes (Keller & Block, 1960; Putnam, 1965). The failure to regenerate thermostability by mixing the separated moieties may be due to some further change in the carbohydrate, or protein, or both. Nevertheless, the variations in enzymic

activity which were observed when the two moieties were mixed indicated that association between them occurred. No information was provided by the present experiments about the nature of bonds between the two moieties of the original complex but the difference between the u.v.-adsorption spectra of the latter and of the protein moiety suggested that aromatic residues of the protein might be involved.

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