Circular-Dichroism Studies on Two β -Lactamases from *Bacillus cereus*

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The circular-dichroism (CD) spectra of β -lactamases I and II from *Bacillus cereus* 569/H are reported, along with that of the β -lactamase II free from carbohydrate. The results show that carbohydrate makes an appreciable contribution to the optical activity of β -lactamase II in the far-ultraviolet, and that removal of carbohydrate greatly affects the optical activity of several aromatic side chains of the protein moiety. Both tyrosyl and tryptophanyl residues are affected, showing that some of these residues must be near to the surface of the protein moiety, close to the site of attachment of the carbohydrate. Although the far-ultraviolet CD spectrum of β -lactamase II resembles that of a protein containing some β -structure, it can be shown that this is a consequence of the optical activity of carbohydrate in this region of the spectrum, and that the protein is likely to contain α -helix rather than β -pleated sheet structure. The overall structures of the protein components of β -lactamases I and II are similar, but not identical, as shown by the dissimilarity of the CD spectra when calculated on a mean residue basis.

The two enzymes, β -lactamase I and β -lactamase II, produced by *Bacillus cereus* 569/H are similar in amino acid composition (Kuwabara, Adams & Abraham, 1970). However, their molecular weights are 27800 and 35600 respectively (Lloyd & Peacocke, 1970), and this difference in molecular weight is at least partly due to the presence of carbohydrate bound to native β -lactamase II (Kuwabara *et al.* 1970), which appears to confer heat-stability to the enzyme (Kuwabara & Lloyd, 1971). Of the two enzymes, only β -lactamase II requires Zn^{2+} as a cofactor (Sabath & Abraham, 1966; Kuwabara, 1970).

It has been shown (Kuwabara & Lloyd, 1971) that native β -lactamase II may be dissociated into two components, one of which contains all of the protein and the other all of the carbohydrate. The protein moiety shows about 65% of the enzymic activity of the native enzyme, but is heat-labile: when the carbohydrate moiety is added to the protein moiety, activity increases. The protein moiety has been designated β -lactamase IIp.

It was decided to study the native conformations of the β -lactamases I and II, and also the conformational changes, if any, which occur in the β -lactamase II when the Zn^{2+} is removed, or when the two components of the enzyme are separated. The present paper gives the results of such a study,

CD* spectroscopy being used to investigate the conformations and conformational changes of the proteins.

MATERIALS AND METHODS

The samples of β -lactamases I and II and the protein and carbohydrate moieties of β -lactamase II were prepared by Dr S. Kuwabara (Kuwabara & Lloyd, 1971). Solutions of the enzymes were dialysed against 1 mM-tris-acetate buffer, pH 7.0, which contains 1 mM- Zn^{2+} for experiments with β -lactamase II (buffer B and BZ, prepared as described by Kuwabara, 1970). The solutions used for the determinations of the CD spectra had extinctions between 0.9 and 1.3 at 280 nm, the extinctions being measured by either a Cary 14 or Unicam SP.800 spectrophotometer.

CD spectra between 350 and 185 nm were measured by means of a Jouan Dichrographe instrument. Cuvettes of different path lengths, namely 10, 5, 2, 1, 0.5 and 0.1 mm, were used to cover different ranges of the spectrum. The trace obtained by measurement with a cuvette of given path length was overlapped by those of both longer and shorter path lengths, to the extent of 40 nm where possible, to allow continuity of measurement. Duplicate spectra were run. Base-lines were determined on the buffer solutions used in the original dialysis. The CD spectra are expressed in terms of $\Delta\epsilon = (\epsilon_l - \epsilon_r)$, the difference in molar extinction coefficients for left- and

* Abbreviation: CD, circular dichroism.

right-circularly polarized light. This measure was used in favour of the more usual ellipticity to allow the similarities of the various β -lactamase II spectra to be pointed out. Conversion into ellipticity per mean residue weight can be made by multiplying by the factor $3300m/M$, where m and M are the molecular weights of mean residue and protein respectively. The ellipticity per residue weight cannot be calculated for β -lactamase II by this method because of the carbohydrate content: the value for M in this case must be taken as that of the protein component, and not that of the intact molecule.

Molar extinction coefficients were estimated for β -lactamases I and IIp by determining the concentrations of solutions of known extinction. The concentrations were measured by determining the refractive index of the solutions on a Brice-Phoenix differential refractometer, after prolonged dialysis against buffer B (0.1M, pH 7.0). Knowledge of the refractive index of the solution relative to that of the solution against which it had been dialysed allowed calculation of the protein concentration (mg/ml; by using $dn/dc = 0.183$), and from this the concentration could be expressed in molar terms from a knowledge of the molecular weights. This knowledge of the molar concentration then allowed calculation of the molar extinction coefficient. The molecular weights used in these calculations were 27800 for β -lactamase I (Lloyd & Peacocke, 1970) and 22500 for β -lactamase IIp (Kuwabara & Lloyd, 1971).

The extinction coefficients determined by this method were 24400 and $24100 \text{ l} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ for β -lactamases I and IIp respectively. The procedure used above cannot be used for determining the extinction coefficient of intact β -lactamase II since the carbohydrate content of the molecule affects the refractive-index increment to an unknown extent, and therefore the molar extinction coefficient was taken to be the same as that of β -lactamase IIp, which contains the protein fraction of the molecule. Although the carbohydrate moiety of the protein, once separated from the IIp portion on Sephadex, showed an apparent absorption (Kuwabara & Lloyd, 1971) this absorption is not necessarily caused by the presence of amino acids bound to the carbohydrate. For, whereas no absorption is caused by the sugar units found in the analysis of this fraction, the absorption appears to arise partly from the presence in the fraction of a quantity of native or denatured β -lactamase II, as shown by the similarity of the amino acid compositions of the intact enzyme and of the carbohydrate moiety. Furthermore, the solution of carbohydrate showed some light-scattering properties caused by the extensive aggregation of carbohydrate: this scattering would result in an apparent absorption of the carbohydrate moiety. It is therefore reasonable to equate the extinction coefficients of β -lactamases II and IIp, since all of the absorption of the protein is caused by the IIp, rather than the carbohydrate moiety.

RESULTS

Figs. 1 and 2 show the near- and far-u.v. CD spectra respectively of β -lactamases I, II and IIp, together with those of zinc-free intact β -lactamase II.

Fig. 1. Near-ultraviolet CD spectra of the β -lactamases. —, β -Lactamase II and Zn^{2+} -free β -lactamase II; ---, β -lactamase IIp; - · - · -, β -lactamase I; · · · · ·, difference β -lactamase II minus β -lactamase IIp (right-hand scale).

Fig. 2. Far-ultraviolet CD spectra of the β -lactamases. —, β -Lactamase II; · · · · ·, Zn^{2+} -free β -lactamase II; ---, β -lactamase IIp; - · - · -, β -lactamase I.

Fig. 3. Difference CD spectra between the β -lactamases II. —, β -Lactamase II minus β -lactamase IIp; ----, β -lactamase II minus Zn^{2+} -free β -lactamase II. The small peak in this difference spectrum at about 220 nm is not significant, and lies in the range of experimental error: the other contributions are beyond the range of experimental error.

Removal of the bound zinc from β -lactamase II causes no alteration of the near-u.v. CD spectrum, but a change is introduced centred about 200 nm in the far u.v. This change appears to be in the form of a single component (Fig. 3). Removal of the carbohydrate moiety of β -lactamase II also results in changes in the CD spectrum at about 200 nm, but in addition there is an alteration in the CD spectrum at about 230 nm, and a profound change in the near-u.v. CD spectrum (Fig. 1).

Of the four molecules studied, only β -lactamases I and IIp show the typical double minimum to be expected from the presence of α -helix. In the CD spectra of native and zinc-free β -lactamase II the double minimum is replaced by a broad minimum at about 215 nm (Fig. 2).

DISCUSSION

The effect of Zn^{2+} on the CD spectrum of β -lactamase II is confined to the far ultraviolet, and therefore primarily reflects changes in the backbone peptide groups of the protein or in the carbohydrate.

The alteration in the far-ultraviolet CD spectrum (Fig. 3) is confined to the region about 200 nm, and therefore is of the wrong shape to represent an alteration in the α -helix or β -pleated sheet content of the protein. This suggests either that the unordered portion of the protein undergoes some small conformational change or that the carbohydrate residues are affected by the removal of the metal ion (Tiffany & Krimm, 1969; Kabat, Lloyd & Beychok, 1969).

β -Lactamases I and II have similar, but not identical, far-ultraviolet CD spectra, but the near-ultraviolet CD difference between the two proteins, and between β -lactamases II and IIp, must be caused by the different environments of tyrosyl and tryptophanyl residues within the proteins. The large difference between β -lactamases II and IIp suggests that many of the side chains are on the surface of the protein fragment, since there is no evidence that a large conformational change occurs to cause the alteration in the surroundings of the aromatic groups. The alteration between β -lactamases II and IIp is therefore caused by the removal of the perturbing effect of carbohydrate, which causes a loss of side-chain optical activity. The inference that the tyrosyl side chains are on the surface of the protein is supported by the slightly higher pK of the tyrosyl residues in β -lactamase II as compared with those of β -lactamase I (Kuwabara *et al.* 1970). The similarity of the shapes of the CD spectra of β -lactamases I and IIp suggests that similar environments exist for the side chains on the two proteins, although the different magnitudes of the CD spectra suggest that different numbers of side chains contribute to the CD spectra of the two molecules.

The two enzymes do not give far-ultraviolet CD spectra that can be fitted adequately by the method of Greenfield & Fasman (1969) to give the amounts of regular structure present. From the value of the CD at 220 nm, estimates of about 25% of α -helix can be made for β -lactamases I, II and IIp. The difference CD spectrum between β -lactamases II and IIp does not suggest that any appreciable helix-coil transition occurs when the carbohydrate is removed, since then the CD difference spectrum would contain a contribution at 220 nm, which is absent in this case (Fig. 3). The difference contribution at 230 nm in Fig. 3 is probably caused by tryptophan (Laiken & Craig, 1969), in agreement with the loss of optical activity in the near-ultraviolet. The loss of tryptophan optical activity will also contribute to the difference CD spectrum at about 220 nm, and further contributions in this region are to be expected from carbohydrate and unordered polypeptide transitions. The carbohydrate component must give a positive CD spectrum to produce the observed effect, and it is

this which masks the 208nm minimum of the polypeptide α -helix, giving a CD spectrum for intact β -lactamase II similar to that of a polypeptide in the β -sheet conformation. This, however, is not the conformation of β -lactamase II, since the change in CD spectrum on removal of the carbohydrate is characteristic neither of a β/α conformational change nor of a β /unordered change.

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