The interaction of anthraquinone dyes with the plasmid-mediated OXA-2 β -lactamase

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(Received 11 March 1982/Accepted 4 May 1982)

Although β -lactamases do not require any nucleotide co-substrates, the OXA-2 type is inhibited competitively by Cibacron Blue 3GA, and by other anthraquinone dyes, including some simpler compounds with no side chain. The enzyme causes a red shift in the spectrum of Cibacron Blue. The β -lactamase can be adsorbed to Blue Sepharose and specifically eluted by benzylpenicillin. These results indicate that the binding of anthraquinone dyes is a specific effect similar to that seen with many nucleotide-binding enzymes.

The ability to produce β -lactamase (penicillin/cephalosporin amido- β -lactamhydrolase; EC 3.5.2.6) is responsible for plasmid-mediated bacterial resistance to penicillins and other β -lactam antibiotics. A variety of plasmid-mediated β -lactamases occurs in Gram-negative bacteria. One class, known as the OXA-2 group and exemplified by the R46 β -lactamase, possess a number of unusual features: not only are these enzymes able to hydrolyse oxacillin rapidly but they are dimeric enzymes, unlike any other β -lactamase so far characterized (Dale & Smith, 1976). Furthermore, they are inhibited by the anthraquinone dye Cibacron Blue (Dale, 1971; Dale & Smith, 1974).

Cibacron Blue is well known as an inhibitor of a number of nucleotide-requiring enzymes; however, the R46 β -lactamase does not require any nucleotide cofactors, nor is there any evidence for nucleotide binding sites. The present study, which forms part of a wider investigation into the structure and function of the R46 β -lactamase, was carried out to determine if the nature of the interaction with Cibacron Blue was similar to that occurring with other, nucleotide-binding enzymes.

A preliminary account of part of this work was presented at the 12th International Congress of Chemotherapy, Florence, July 1981.

Materials and methods

Chemicals and dyes

Benzylpenicillin was obtained from Glaxo (as the sodium salt) or from Sigma (potassium salt). Ciba-

cron Blue 3GA was a gift from Ciba-Geigy, Remazol Brilliant Blue R from Hoechst, Procione Blue MX-R, Procione Blue H-GR and Nylomine Blue B-3G from ICI. 1-Amino-4-(4'-aminophenylamino)anthraquinone-2,3'-bis-sulphonic acid was donated by Sandoz. Solway Ultra Blue, Alizarin Saphirol, Solway Blue SE and Alizarin Cyanine Green were purchased from Gurr, Erythrosin B from Sigma, Alizarin Red S from BDH and bromaminic acid from Pfaltz and Bauer. Other anthraquinone derivatives were obtained from Aldrich. The structures of some of these dyes are shown in Fig. 1, and the Colour Index numbers and generic names are listed in Table 1. Blue Sepharose CL-6B was obtained from Pharmacia.

Purification of the R46 β -lactamase

The enzyme was purified from an extract of *Escherichia coli* J62 (R46) (laboratory no. JD210) as described by Dale & Smith (1971). The enzyme preparations used had a specific activity in excess of 90 units/mg of protein. Enzyme activity was determined by the hydroxylamine assay (see below) and the protein concentration by the method of Waddell (1956).

β -Lactamase assays

The hydroxylamine assay (Dale & Smith, 1971) was used to determine the activity of the enzyme preparation. One unit of enzyme catalyses the hydrolysis of 1 μ mol of benzylpenicillin/min at 30°C in 0.025 M-sodium phosphate buffer, pH 7.4.

For determination of K_m and K_i values, the microiodometric assay method was used, with benzylpenicillin as substrate, as described by Dale & Smith

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R =

Cibacron Blue 3GA

Procion Blue H-GR

Procion Blue MX-R

ASSO

Solway Ultra Blue

Remazol Brilliant Blue R

Nylomine Blue B-3G

Alizarin Saphirol

Solway Blue SE

Alizarin Red S

Fig. 1. Structures of the anthraquinone dyes used

Abbreviation: ASSO, 1-amino-4-(4'-aminophenylamino)anthraquinone-2,3'-bis-sulphonic acid.

(1971). With some dyes, especially at high concentration, some decolourization of the starch-iodine was observed in the absence of enzyme. Inclusion of the dye in the blank cuvette compensated for this effect, but where the non-enzymic rate was significant the results have not been included.

For this reason, confirmation of some of the results was attempted by using a u.v.-absorption assay (Samuni, 1975) with benzylpenicillin or cephaloridine as the substrate and measuring the decline in A_{240} or A_{255} . However, the relatively high concentration of substrate makes the u.v. assay unsuitable for determination of inhibition constants.

The K_m value for benzylpenicillin was determined by means of double-reciprocal (Lineweaver-Burk) plots in the absence of inhibitor. The value obtained ($4.7 \mu\text{M}$; 12 determinations; s.e.m. = 0.19) is very close to the previously published value of $4.2 \mu\text{M}$ (Dale & Smith, 1971). The K_i values were calculated from double-reciprocal plots in the presence of a fixed concentration of inhibitor by using the expression:

$$K_i = \frac{i}{(K_p/K_m) - 1}$$

where $1/K_p$ is the negative intercept on the $1/s$ axis. Repeat determinations at different inhibitor concentrations gave consistent values of K_i .

Results

The double-reciprocal plots obtained with different concentrations of Cibacron Blue as inhibitor showed a typically competitive pattern (Fig. 2). This was also true of the other anthraquinone dyes that were effective inhibitors. The K_i values determined

Fig. 2. Inhibition of the R46 β -lactamase by Cibacron Blue 3GA

Reaction rates, using various concentrations of the substrate (benzylpenicillin), were determined by the micro-iodometric method in the absence (○) or the presence of Cibacron Blue at $2.4 \mu\text{M}$ (Δ) and at $7.1 \mu\text{M}$ (□).

from these plots (Table 1) show that extensive variations in the nature of the substituent on the C-4 amino group of the anthraquinone nucleus had little effect on the affinity of the dye for the enzyme, and also that some simple anthraquinone compounds with no side chain were able to act as equally effective inhibitors. However, varying the nature of the substituents on the nucleus itself had a marked effect in some cases. Thus, although Alizarin Saphirol was an effective inhibitor, Solway Blue SE (with a very similar structure; see Fig. 1) was not. The u.v. assay method confirmed that Solway Blue SE was a much less effective inhibitor

Table 1. *Inhibition of R46 β -lactamase by anthraquinone derivatives*
 Abbreviation: ASSO, 1-amino-4-(4'-aminophenylamino)anthraquinone-2,3'-bis-sulphonic acid.

Commercial name	Colour-index generic name	Colour index number	K_i (μM)
Cibacron Blue 3GA	Reactive Blue 2	61 211	1.2*
Procion Blue H-GR	Reactive Blue 5	61 205 : 1	2.7
Procion Blue MX-R	Reactive Blue 4	61 205	1.0
ASSO	—	—	4.0
Solway Ultra Blue	Acid Blue 25	62 055	1.0
Remazol Brilliant Blue R	Reactive Blue 19	61 200	0.5
Nylomine Blue B-3G	Acid Blue 40	62 125	2.7
Alizarin Saphirol	Acid Blue 45	63 010	1.2
Solway Blue SE	Acid Blue 43	63 000	>20
Alizarin Red S	Mordant Red 3	58 005	3.0
1-Aminoanthraquinone-2-sulphonic acid	—	—	>20

* Six determinations (S.E.M. = 0.26).

than Alizarin Saphirol or Cibacron Blue; although inhibition could be demonstrated by this method, it was not possible to assign K_i values.

The 1-amino-2-sulphonic acid was weakly inhibitory, but the 2-sulphonic acid and the 2,6-bis-sulphonic acid showed no detectable inhibition. Other derivatives that failed to inhibit include bromaminic acid, Alizarin Cyanine Green and 1,8-dihydroxyanthraquinone. Erythrosin B (Acid Red 51; CI 45430), which is not an anthraquinone dye (although it has some structural similarity), also showed some inhibitory effect in the u.v. assay (result not shown).

Thompson & Stellwagen (1976) observed that lactate dehydrogenase caused a red shift in the absorption spectrum of Cibacron Blue, with a maximum value of the difference spectrum at 660 nm. A similar phenomenon has since been observed with a number of other enzymes to which Cibacron Blue binds. The R46 β -lactamase causes a similar red shift with a maximum at 680 nm and a less well defined minimum at around 580 nm (Fig. 3). This indicates that the form of binding in the present case may be similar to that observed with lactate dehydrogenase and other nucleotide-binding enzymes.

Further information on the interaction of proteins with Cibacron Blue comes from the use of Blue Sepharose (Cibacron Blue immobilized on a Sepharose matrix), which also provides a form of affinity chromatography for rapid purification of these proteins. A sample of the crude β -lactamase was therefore applied to a Blue Sepharose column. After removing non-adsorbed material by washing with buffer, elution of the enzyme was achieved with a solution of benzylpenicillin (10 mg/ml; 28 mM). Table 2 shows that this procedure, in a single step, resulted in a specific enzyme activity as high as

Fig. 3. *Difference spectra of Cibacron Blue in the presence of R46 β -lactamase*

The sample cuvette contained the enzyme (2.5 μM). Cibacron Blue (7 μM ; curve A; 34 μM ; curve B) was added to both cuvettes.

that of the purified β -lactamase obtained by the more lengthy procedure of Dale & Smith (1971), although only 15% of the enzyme was recovered from the column. The enzyme could also be eluted by NaCl, although only at a very high concentration (1.2 M), which is strongly inhibitory to the enzyme. Elution could not be achieved with low concentrations of ATP (5 mM), NADH (10 mM) or FMN (10 mM). These results confirm that the binding of Cibacron Blue is not merely a non-specific interaction as is seen with some proteins (Stellwagen, 1977), and also lend support to previous observations (J. W. Dale, unpublished work) that this enzyme does not appear to have any nucleotide-binding capacity. Ogawara & Horikawa (1979) were

Table 2. *Purification of the R46 β -lactamase by affinity chromatography on Blue Sepharose CL-6B*

A sample of crude enzyme was applied to a column (10 cm $\times$ 0.9 cm) of Blue Sepharose CL-6B equilibrated with 10 mM-sodium phosphate, pH 7.4. After washing the column with the same buffer, the enzyme was eluted with a solution of benzylpenicillin (10 mg/ml) also in the same buffer.

	Volume (ml)	Protein (mg)	Enzyme activity (units)	Specific activity (units/mg)
Sample applied	5	70	106	1.5
Material eluted	1	0.11	15.6	142

able to purify the β -lactamase from *Streptomyces cellulosae* by using Blue Sepharose, and were also able to demonstrate some degree of interaction of that enzyme with NADP⁺.

Discussion

Thompson *et al.* (1975) and Thompson & Stellwagen (1976) proposed that Cibacron Blue (or the derivative Blue Dextran) binds specifically to proteins containing a 'dinucleotide fold', due to the conformational relationships between the dye and, for example, NAD⁺. These conformational relationships involved not only the anthraquinone moiety but also the terminal phenylsulphonate ring. Other workers (e.g., Beissner & Rudolph, 1978; Edwards & Woody, 1979) have shown that the interaction is not in fact specific for the dinucleotide fold, nor does it depend on specific conformational relationships. However, it remains true that most of the enzymes for which a specific interaction with Cibacron Blue has been demonstrated do have a binding site for nucleotides such as NAD⁺, NADP⁺ or ATP, although Ramesh & Rao (1980) reported the interaction of Cibacron Blue with the tetrahydrofolate-binding domain of an enzyme (serine hydroxymethyltransferase) that does not utilize a nucleotide co-substrate. The present study confirms that Cibacron Blue can interact with enzymes that do not have a nucleotide-binding site.

Ashton & Polya (1978) showed that a wide range of sulphonated aromatic dyes were able to inhibit cyclic nucleotide phosphodiesterase and lactate dehydrogenase. Bornmann & Hess (1977) and Beissner & Rudolph (1978) concluded that the anthraquinone and neighbouring phenylsulphonate

rings were sufficient for inhibition of a variety of nucleotide-binding enzymes. The results reported in the present paper show that, for the R46 β -lactamase at least, simple anthraquinone derivatives can act as effective inhibitors.

Part of this work was supported by the Science Research Council and Beecham Pharmaceuticals under the Co-operative Awards in Science and Engineering scheme.

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