The isolation of a peptide from the catalytic domain of *Bacillus stearothermophilus* tryptophyl-tRNA synthetase

The interaction of Brown MX-5BR with tyrosyl-tRNA synthetase

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Tryptophyl-tRNA synthetase is irreversibly inactivated by Procion Brown MX-5BR with an apparent dissociation constant (K_D) of $8.8 \mu\text{M}$ and maximum rate of inactivation k_3 0.192 s^{-1} . The specificity of the interaction is supported by two previously reported observations. Firstly, Brown MX-5BR inactivation of tryptophyl-tRNA synthetase is inhibited by substrates, and, secondly, the aminated derivative of Brown MX-5BR is a competitive inhibitor of tryptophyl-tRNA synthetase with a K_i of $2 \times 10^{-4} \text{ M}$ with respect to both tryptophan and ATP. Tryptic digestion of the dye-affinity-labelled enzyme and subsequent resolution of the peptides by h.p.l.c. yielded one major dye-peptide peak. Amino acid sequence analysis resulted in the identification of the dye-binding domain centred on lysine-178. Tyrosyl-tRNA synthetase is also inactivated by Procion Brown MX-5BR, and this inactivation is prevented by ATP but not by tyrosine. The interaction of tyrosyl-tRNA synthetase with hydroxylated Brown MX-5BR exhibited non-competitive kinetics with respect to the amino acid-binding site and competitive kinetics against ATP with a K_i of $6 \times 10^{-6} \text{ M}$.

INTRODUCTION

Procion dyes are a large family of commercially available organic dyes, the most widely studied of which is Cibacron Blue F3G-A. This dye has been shown to interact specifically, but not exclusively, with nucleotide-requiring enzymes at the dinucleotide fold (Stellwagen, 1977; Biellman *et al.*, 1979; Edwards & Woody, 1979). It is only more recently, however, that the potential of the whole family of dyes, which number approximately 80, as affinity probes and labels of enzymes has been fully appreciated. This is exemplified by the inactivation of horse liver alcohol dehydrogenase by Procion Blue MX-R and the subsequent identification of the labelled peptide in the NAD^+ -binding site of this protein (Small *et al.*, 1982). Similarly, carboxypeptidase G inactivated with Procion Red MX-8B, a dye shown to be a competitive inhibitor of this enzyme, identified threonine-279 at the dye-binding site (Hughes *et al.*, 1984). Clonis & Lowe (1980) have shown that Procion Green H-4G will specifically inactivate yeast hexokinase and that this inactivation can be prevented by substrates.

Aminoacyl-tRNA synthetases (XTSs) play a central role in protein synthesis and are capable of recognizing three substrates, correctly attaching corresponding amino acids (AA) to their cognate tRNAs (Ofengand, 1978):

These enzymes therefore contain highly discriminating binding sites.

Specific probes for any of the substrate-binding sites of XTSs are rare. Amino acid derivatives modified at the carboxy function have been shown to be inhibitory (Calendar & Berg, 1966), whereas modification at the amino function abolishes binding. Modification of L-isoleucyl-tRNA synthetase with L-isoleucylbromomethane has been achieved (Rainey *et al.*, 1977), and of L-tryptophyl-tRNA synthetase (WTS) with L-tryptophylchloromethane (Kisselev *et al.*, 1979). The precise reacting residue has not been identified in either case. Bruton & Hartley (1970) isolated an octapeptide from methionyl-tRNA synthetase labelled with *p*-nitrophenyl-oxycarbonylmethionyl-tRNA and identified a lysine residue from within the methionyl-tRNA binding site. More recently, a cysteine residue within the β -subunit of phenylalanyl-tRNA synthetase was specifically labelled when 3'-oxidized tRNA was used (Renaud *et al.*, 1982).

Elution characteristics of XTSs from various triazine dye-Sepharose columns (Bruton & Atkinson, 1979) have indicated that different dyes can mimic specific amino acids or ATP and other dyes the tRNA substrates. In particular, tryptophan was excellent for elution of essentially homogeneous WTS from a Procion Brown MX-5BR-Sepharose column. The same column bound tyrosyl-tRNA synthetase (YTS) strongly, with significant amounts of enzyme being eluted by both tyrosine and ATP.

Most of the crystal structure of native YTS is well defined (Bhat *et al.*, 1982), and the binding sites of amino acid and ATP are well delineated. The crystal structure of WTS is as yet unknown, although work is proceeding (Coleman & Carter, 1984; Carter & Coleman, 1987).

Abbreviations used: YTS, tyrosyl-tRNA synthetase (EC 6.1.1.1); WTS, tryptophyl-tRNA synthetase (EC 6.1.1.2); XTS, aminoacyl-tRNA synthetase (in these three abbreviations the first letter designates the amino acid specificity in the single-letter code for amino acids).

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There has been no success, however, in identifying catalytically important residues of WTS or YTS by affinity-labelling techniques. Although several examples of active-site probing and labelling exist in the literature, with respect to WTS neither the amino acid sequence of the peptides nor the identity of the labelled residues have been reported.

The above facts prompted studies on the interaction of Procion Brown MX-5BR with WTS and YTS and to investigate the use of Brown MX-5BR as a novel probe of WTS.

MATERIALS AND METHODS

Materials

Homogeneous WTS and YTS from *Bacillus stearothermophilus* (EC 6.1.1.2 and EC 6.1.1.1 respectively), of specific activities approx. 1140 units/mg and 150 units/mg respectively were purified as described by Atkinson *et al.* (1979). ATP (disodium salt) was obtained from Boehringer Corp. (Lewes, East Sussex, U.K.) and L-amino acids were from Sigma Chemical Co. (Poole, Dorset, U.K.). $\text{Na}_4^{32}\text{P}_2\text{O}_7$ was purchased from Amersham International (Amersham, Bucks., U.K.). Procion Brown MX-5BR was a gift from Dr C. V. Stead (ICI Organics Division, Blakely, Manchester, U.K.); its structure is shown in Fig. 1. Pure Procion Brown MX-5BR used in affinity-labelling and inactivation/protection experiments was prepared by using preparative silica 60 t.l.c. plates, obtained from BDH Chemicals (Poole, Dorset, U.K.), which were developed in 2-methylpropan-1-ol/propan-1-ol/ethyl acetate/water (2:4:1:3, by vol.). Partially purified Procion Brown MX-5BR was prepared by precipitation in 0.3 M-HCl, collected by centrifugation at 10000 g for 30 min and dried on Whatman no. 54 filter papers. Peptides used in the determination of the percentage recoveries of dyed material from a C_{18} μ Bondapak column were obtained from Sigma Chemical Co. and Cambridge Research Biochemicals (Cambridge, U.K.).

Assay of XTS activities

WTS and YTS activities were measured by using the ATP/pyrophosphate exchange assay (Heinrikson & Hartley, 1967). Assay tubes contained, in a final volume of 200 μ l, 20 μ mol of Tris/HCl buffer, pH 8.0, 2 μ mol of KF (pyrophosphatase inhibitor), 1 μ mol of MgCl_2 , 0.4 μ mol of ATP, 0.4 μ mol of $\text{Na}_4^{32}\text{P}_2\text{O}_7$ (specific radioactivity 80000–150000 c.p.m./ μ mol), 0.5 μ mol of

amino acid and 0.1 μ mol of 2-mercaptoethanol. Bovine serum albumin (0.5 μ mol) was normally included in assays of XTS activities; however, it was omitted in inhibitor studies, as many dyes bind to bovine serum albumin.

Inactivation and protection studies

Inactivation studies were carried out in 200 μ l total volume at 37 °C. The incubation mixtures contained in 100 mM-Hepes/NaOH buffer, pH 8.5, 50 or 100 nmol of pure or partially purified dye plus substrates or other amino acids at the concentrations indicated. XTS was added at time zero, and samples were taken and assayed for enzyme activity, in duplicate, at various intervals.

Determination of inhibitor constant of hydroxylated Procion Brown MX-5BR for YTS

Inhibitor constants of unreactive, aminated or hydroxylated dye with respect to tyrosine were measured in 200 μ l total volume of assay mix with MgATP (MgCl_2 and ATP in 2.5:1 ratio) as saturating fixed substrate (2 mM). The inhibitor concentration was varied with a series of amino acid concentrations around the K_m for tyrosine.

The inhibitor constant of the dye with respect to MgATP was determined in 400 μ l total volume assay mix, with tyrosine as fixed saturating substrate (2 mM). Inhibitor concentrations were varied with a series of MgATP concentrations from the K_m upwards. A higher specific radioactivity of $\text{Na}_4^{32}\text{P}_2\text{O}_7$ at 350000–500000 c.p.m./ μ mol and slightly smaller amounts of enzyme were used in these experiments. This was necessary in order to keep the percentage of total measured radioactivity in the assay below 10% of that available, as ATP is used up and not regenerated.

Affinity labelling of WTS by Procion Brown MX-5BR

Homogeneous WTS of high specific activity was dialysed against N_2 -gassed 20 mM-Hepes/NaOH buffer, pH 8.5. The protein concentration was calculated from the absorption coefficient $A_{1\text{ cm}, 280}^{0.1\%} = 1.32$. The concentration of freshly prepared pure Brown MX-5BR was measured by using the molar absorption coefficient $\epsilon_{530} = 1.56 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$. At time zero a portion of dye was added to the enzyme to give a dye/enzyme molar ratio of 0.25:1 and the mixture was incubated at 37 °C for 30 min. At this time a second portion of dye was added and the mixture was incubated as before. This procedure was repeated six times, after which the percentage of the initial enzyme activity remaining was less than 5%.

Residual free dye was removed from labelled enzyme by passage through a Sephadex G-50 (fine grade) column (bed volume 15 ml, height 7 cm) equilibrated in water.

A tryptic digestion of the labelled WTS was performed at a trypsin/WTS ratio of 1:200 (w/w) at 37 °C for 4 h. The digestion was facilitated by prior heat denaturation of WTS at 70 °C for 5 min in 5 mM-Tris/HCl buffer, pH 8.0.

Isolation and purification of Procion Brown MX-5BR-labelled peptides

Peptides were resolved by h.p.l.c. with a C_{18} μ Bondapak reverse-phase column (250 mm $\times$ 4.6 mm) and a linear gradient of 5–60% (v/v) acetonitrile in 10 mM-ammonium acetate buffer, pH 6.8, over 1 h (Waters

Fig. 1. Planar representation of the half-molecule of Brown MX-5BR

Brown MX-5BR is a dimer with the halves centred around the Cr^{2+} ion. X = Cl for the reactive dichlorotriazine dye; X = OH for hydroxylated Brown MX-5BR; X = NH_2 for aminated Brown MX-5BR.

Fig. 2. Inactivation of WTS by pure and partially purified Brown MX-5BR and protection by substrates

Inactivation tubes contained $2.5 \mu\text{M}$ -WTS plus $250 \mu\text{M}$ pure ($\diamond$, $\triangle$, and $\circ$) or partially purified ($\blacklozenge$, $\blacktriangle$ and $\bullet$) Brown MX-5BR in $200 \mu\text{l}$ of 100 mM -Hepes buffer, pH 8.5. Tubes were incubated at 37°C and contained dye (inactivation control; $\circ$ and $\bullet$), no dye ($\blacktriangledown$), dye plus 1 mM -tryptophan ($\diamond$ and $\blacklozenge$), or dye plus 1 mM -ATP ($\triangle$ and $\blacktriangle$).

Associates HPLC system). Where the elution profile indicated that the dyed peptides were unlikely to be sufficiently pure for sequence analysis, samples were rechromatographed on an extended 2 h gradient or on a DuPont C_8 Zorbax column ($250 \text{ mm} \times 4.6 \text{ mm}$) with a convex gradient over 1 h.

Fig. 3. Inactivation of YTS by Brown MX-5BR and protection by substrates

Inactivation tubes contained $0.5 \mu\text{M}$ -YTS plus $500 \mu\text{M}$ -Brown MX-5BR in $200 \mu\text{l}$ of 100 mM -Hepes buffer, pH 8.5. Tubes were incubated at 37°C and contained dye (inactivation control; $\square$), no dye ($\circ$), dye plus 5 mM -tyrosine ($\triangle$); or dye plus 5 mM -ATP ($\bullet$).

Amino acid sequence analysis of dyed samples

Dyed samples were analysed using the Applied Biosystems gas-phase protein sequencer (Hunkapiller *et al.*, 1983). Amino acid phenylthiohydantoin derivatives were identified by comparison with amino acid phenylthiohydantoin standards after resolution by h.p.l.c. with a reverse-phase cyanopropyl column (Hunkapiller & Hood, 1983).

Determination of percentage recoveries of dyed peptides

Various peptides of amino acid chain lengths ranging from three to 22 residues were dyed with pure Brown MX-5BR at a 10:1 molar ratio of peptide over dye concentration. A $1 \mu\text{mol}$ portion of each peptide was dissolved in $200 \mu\text{l}$ of 20 mM -Hepes/NaOH buffer, pH 8.5, mixed with 100 nmol of pure Brown MX-5BR and incubated at 60°C for 1 h. Then 4 nmol of each dyed peptide, estimated from the molar absorption coefficient of pure Brown MX-5BR, was loaded on to a C_{18} $\mu\text{Bondapak}$ column and eluted with a linear gradient of 5–60% (v/v) acetonitrile in 10 mM -ammonium acetate

Fig. 4. Inhibition of YTS by hydroxylated Brown MX-5BR

YTS (0.04 nmol) was assayed in $400 \mu\text{l}$ volumes of assay buffer containing high-specific-radioactivity $\text{Na}_4^{32}\text{P}_2\text{O}_7$ at $420000 \text{ c.p.m.}/\mu\text{mol}$, with tyrosine at the fixed saturating substrate concentration of 2 mM and a range of ATP concentrations from $50 \mu\text{M}$ to $500 \mu\text{M}$. Assay mixtures contained no dye ($\bullet$), 0.33 mM -dye ($\circ$), or 0.66 mM -dye ($\blacksquare$).

buffer, pH 6.8, over 60 min. The eluate was monitored at 214 nm and 530 nm, and the percentage recovery of dyed peptides was calculated by using the absorption coefficient of Brown MX-5BR. A 4 nmol portion of a tryptic digest of Brown MX-5BR-labelled WTS (also estimated from dye concentration) was chromatographed in the same way and the percentage recoveries of all dyed material were compared.

RESULTS

Inhibitor studies

Because of the difficulties in purifying large amounts of pure Brown MX-5BR and the inherent instability of the purified dye, a comparative study was made of the inactivating abilities of partially purified Brown MX-5BR and the freshly prepared major coloured component (approx. 80%) of the dye purified by t.l.c. The inactivating ability of pure Brown MX-5BR with respect to WTS has been reported previously (McArdell *et al.*, 1982).

Fig. 2 shows the inactivation of WTS by partially purified and pure Brown MX-5BR and the protection afforded by tryptophan and ATP. There was no significant difference observed between the interactions of both dye preparations with WTS. Tryptophan and ATP protected WTS from inactivation by Brown MX-5BR purified by t.l.c. or partially purified by acid precipitation to the same relative extents.

Fig. 3 shows that tyrosine does not protect YTS from inactivation by Brown MX-5BR, whereas ATP confers significant protection to this enzyme. The inhibition constant of aminated Brown MX-5BR with respect to WTS reported previously (McArdell *et al.*, 1982) showed that the dye has a high affinity for the tryptophyl-adenylate-binding site. The inhibition of YTS by aminated Brown MX-5BR exhibited mixed kinetics and a low affinity for the tyrosine-binding site. The hydroxylated dye, however, is a non-competitive in-

hibitor of YTS with respect to tyrosine with a K_i of 2×10^{-3} M but a competitive inhibitor with respect to ATP with a K_i of 6×10^{-5} M, as shown in Fig. 4. Hydroxylated Brown MX-5BR therefore has a high affinity for the ATP-binding site of YTS, approximately one order of magnitude higher than that of ATP.

Affinity labelling of WTS by Procion Brown MX-5BR

Inhibition of WTS by aminated Brown MX-5BR, the substrate protection data and the evidence that the reactive dye has a high affinity for the enzyme, with a K_D of approx. 6×10^{-6} M, suggested that Brown MX-5BR would be a specific affinity label of the tryptophyl-adenylate-binding site of WTS.

A 21 mg (567 nmol) portion of homogenous WTS was therefore inactivated with pure Brown MX-5BR as described in the Materials and methods section, 95% of the initial enzyme activity being lost after 2 h incubation at 37 °C at pH 8.5. The stoichiometry of dye binding was determined after removal of free dye by gel filtration on Sephadex G-50 (fine grade). Spectroscopic measurements of the labelled enzyme in 8 M-urea gave a binding ratio of 0.92 mol of dye/mol of enzyme subunit (M_r 37000).

H.p.l.c. resolution of a tryptic digest of the heat-denatured Brown MX-5BR-labelled WTS is shown in Fig. 5. It may be noted that Brown MX-5BR-labelled WTS is more resistant to proteolysis by trypsin than is native WTS. Even after heat denaturation of the enzyme, the fingerprint of the tryptic peptides from dyed WTS was significantly different from that obtained from native WTS, as can be seen in Fig. 6. This suggests that the large dye molecule protects the peptide bonds adjacent to lysine and/or arginine residues from tryptic cleavage, or perhaps that the large dye chromophore can inhibit trypsin even though it is covalently bound to inactivated WTS. Four dyed samples were collected from the resolved peptides (see Fig. 5, lower curve). Fraction 4 was sufficiently pure for automated amino acid sequence analysis; fractions 1–3, however, contained unacceptably high amounts of contaminating material and were

Fig. 5. Resolution of tryptic peptides of Brown MX-5BR-labelled WTS by reverse-phase h.p.l.c.

A 1.2 mg portion of a tryptic digest of Brown MX-5BR-labelled WTS was injected on to a reverse-phase C_{18} column equilibrated with 5% (v/v) acetonitrile in 10 mM-ammonium acetate buffer, pH 6.8. The column was run isocratically at 1 ml/min and 8.4 MPa (1200 lbf/in²) for 15 min. Peptides were resolved with a linear gradient of 5–60% (v/v) acetonitrile in the same buffer as above over a further 60 min.

Fig. 6. Resolution of tryptic peptides of WTS by reverse-phase h.p.l.c.

A 150 μ g portion of a tryptic digest (WTS/trypsin ratio 200:1, w/w; 4 h; 37 °C) of heat-denatured WTS (70 °C for 5 min in 10 mM-Tris/HCl buffer, pH 8.0) was injected on to a reverse-phase C_{18} column equilibrated with 5% (v/v) acetonitrile in 10 mM-ammonium acetate buffer, pH 6.8. The column was run isocratically at 1 ml/min and 8.4 MPa (1200 lbf/in²) for 10 min. Peptides were eluted with a linear gradient of 5–60% (v/v) acetonitrile in 10 mM-ammonium acetate buffer, pH 6.8, over a further 60 min.

further purified. Fractions 2 and 3 were satisfactorily purified by using an extended 2 h gradient on the C_{18} column. Purification of fraction 1 was achieved by using a Dupont C_8 Zorbax column (250 mm $\times$ 4.6 mm) with a convex gradient over 1 h. Pure fractions 1, 2 and 3, however, gave no *N*-termini, and were possibly degradation products of the dye. Continued Sequenator cycles produced only background traces of amino acids. Amino

acid analyses of the samples, carried out with a Biotronik LC5000 amino acid analyser, showed that negligible amounts of amino acids were recovered from loadings of up to 4 nmol of dyed material.

The amino acid sequence of fraction 4 was determined to be:

Ile-Pro-Xaa-Val-Gly-Ala-Arg

This corresponds to the unique sequence of amino acid residues 176–182 in the amino acid sequence of WTS (Hall *et al.*, 1982), with the unknown, Xaa, in the determined sequence being lysine-178.

A small fraction of the digested dyed WTS previously separated from the bulk of the material was digested for a further 72 h and chromatographed under the same conditions as described previously. The relative peak heights changed; the recovery of peak 4 was considerably diminished, fraction 2 became the major species and new peaks began to appear. Not enough material was available for further sequence analysis, but, as fractions 2 and 4 appeared to be interchangeable and it had previously been established that fraction 2 contained no peptide material, it was concluded that fractions 1–3 (see Fig. 5) were dye breakdown products and/or 'stacked' dye material that had not covalently reacted with WTS and/or single dyed lysine residues.

Percentage recoveries of all dyed peptides studied from a C_{18} reverse-phase column varied between 50 and 100%. Total recovery of hydroxylated Brown MX-5BR was achieved, as can be seen in Table 1, although only 12% of the dyed material of the tryptic digest of Brown MX-5BR-labelled WTS was recovered; the significance of this is discussed below.

DISCUSSION

Commercially available triazine dyes, when marketed, contain a number of additives to improve their handling

Table 1. Percentage recoveries of Brown MX-5BR-labelled peptides from a C_{18} μ Bondapak column

Peptides were dyed as described in the Materials and methods section. Elution from a reverse-phase C_{18} μ Bondapak column (250 mm $\times$ 4.6 mm internal diam.) was achieved with a linear gradient of 5–60% (v/v) acetonitrile in 10 mM-ammonium acetate buffer, pH 6.8, over 60 min. The flow rate was 1 ml/min. Eluted peptides were monitored at 214 nm and 530 nm.

Dyed material	Recovery (%)
Hydroxylated Brown MX-5BR	100
Cys	89
Glu-Cys-Gly	100
Gly-Gly-Tyr-Arg	49
Gly-His-Arg-Pro	75
Asp-Arg-Val-Tyr-Ile-His-Pro-Phe (angiotensin II)	58
Glu-Glu-Arg-Leu-Gly-Asp-Glu-Trp-Ala-Val-Gly-His-Leu-Met (bombesin)	64
Corticotropin-(18–39)-peptide (human clip)	88
Tryptic digest of Brown MX-5BR-labelled WTS	12

and storage properties. For the purpose of these experiments these additives were routinely removed by acid precipitation. Procion Brown MX-5BR that has been partially purified in this way is stable for several weeks. For affinity-labelling studies, a few milligrams of pure Brown MX-5BR were prepared by t.l.c. as described in the Materials and methods section. The main band (approx. 80%) of Brown MX-5BR, however, is relatively unstable, owing to autocatalytic hydrolysis of the reactive triazine moiety, and the pure dye must be used within 1 day of its preparation. For ease of handling and to avoid possible problems by using an unstable pure dye, a comparison was made between the interaction of WTS with pure and partially purified Brown MX-5BR. No significant difference was observed, and therefore the most satisfactory approach was to use partially purified Brown MX-5BR for kinetic studies.

In contrast with WTS, no protection from inactivation by Brown MX-5BR was afforded to YTS by the cognate amino acid. This indicates that Brown MX-5BR binds to WTS and YTS in different modes at different sites. Hydroxylated Brown MX-5BR was shown to be a non-competitive inhibitor at the tyrosine-binding site of YTS and a competitive inhibitor with high affinity at the ATP-binding site. The fact that Brown MX-5BR discriminates quite clearly between the tyrosine-binding and MgATP-binding sites is interesting in comparison to the observed behaviour of these two substrates in crystals of YTS. When either ATP or tyrosine is soaked into crystals of YTS, both ligands bind in the tyrosine cleft (Monteilhet *et al.*, 1984), but when ATP and tyrosine are soaked in together, tyrosyladenylate is formed *in situ* and the AMP moiety occupies a new site. The observed tyrosine-binding site of YTS is a cleft 1.0 nm (10 Å) deep surrounded by protein on all sides except for a narrow access channel. The adenylate portion of tyrosyladenylate, however, binds in a more accessible cup-like depression of the β -sheet. It is therefore probable that Brown MX-5BR is sterically excluded from the tyrosine-binding site but not the ATP-binding site. Monteilhet *et al.* (1984) have observed, in the crystal structure of YTS, that, unless adenosine is covalently linked to a ligand such as tyrosine, which is firmly bound in a defined orientation, adenine is not sufficiently strongly bound to YTS to appear in the electron-density difference maps of that area of the molecule. It can therefore be concluded that, although tyrosine is adenylated in YTS crystals with high precision and fidelity, it does so without strongly orientating the ATP molecule. This lends support to the idea that the ATP-binding site of YTS is a much more open structure than the tyrosine-binding site with respect to dye binding.

The affinity-labelling of WTS by Brown MX-5BR was shown to be specific. Only one residue, lysine-178, was covalently labelled by Brown MX-5BR. The low percentage recovery of dyed peptides 1, 2, 3 and 4 (Fig. 5), which were purified before sequence analysis, appears not to be due to any intrinsic property of the individual peptides. Recoveries of dyed peptides studied individually suggest that Brown MX-5BR does not bind strongly to the C_{18} matrix, all peptides being eluted in the first half of the gradient in reasonably high yield. The fact that only 12% of the loaded Brown MX-5BR was recovered when the sample applied was a digest of Brown MX-5BR-labelled WTS suggests that incomplete digestion is the

cause for large losses of material at this point. Undigested material will bind to the top of the guard column and remain there. The significant differences observed between the yields and the fingerprints of native versus Brown MX-5BR-labelled WTS also indicate that this may be the case.

In the predicted secondary structure of *Escherichia coli* WTS (Hall *et al.*, 1982), lysine-181 lies in the same relative position and secondary structure area as lysine-178 of the *B. stearothermophilus* enzyme. The surrounding amino acid residues of the isolated active-site peptide are identical in both enzymes except for the replacement of serine-182 in the *E. coli* enzyme by valine in the *B. stearothermophilus* enzyme. It seems therefore likely that the primary and secondary structures of the two proteins have been highly conserved in this area.

Lysine residues have been implicated in the mechanism of action of XTSs by various authors (Bruton & Hartley, 1968; Lotfield, 1972; Bosshard *et al.*, 1978; Bruton, 1979; Fayat *et al.*, 1978; Winter *et al.*, 1979).

Lysine-178 of WTS lies on the C-terminal side of a proposed 15-residue α -helix followed by a short (five-residue) random coil, which in turn is followed by a β -sheet consisting of eight residues terminated by isoleucine-176 (Hall *et al.*, 1982). Lysine-178 is the last in a short region before the chain undergoes a reversal of direction. This region of WTS contains distinct similarities with the region around lysine-225 of YTS. In the latter, the preceding α -helix consists of 17 residues, followed by a short random coil of six residues and then a β -pleat containing six residues, terminated by proline-221. Lysine-225 is the fourth residue downstream of this. This area is important in YTS. Lysine-225 in the latter and indeed lysine-178 in WTS precede a lysine- and arginine-rich area, the lysine residues of which have been implicated in tRNA binding in YTS (Bosshard *et al.*, 1978). Lysine-225 in YTS is in fact the first lysine residue in the YTS structure implicated in this interaction. In YTS also the C-terminal continuation of the β -pleat (the linking peptide) immediately preceding lysine-225 makes contact with the adenine moiety of ATP (Bhat *et al.*, 1982). This similarity in structure between these proteins in this domain may account for the competitive interactions between Brown MX-5BR and both enzymes.

A general method for the systematic replacement of amino acid residues in an enzyme has emerged as an alternative method for the direct analysis of their molecular roles in substrate binding or catalysis, 'site-directed mutagenesis' (Dalbadie-McFarland *et al.*, 1982; Fersht *et al.*, 1985; Jones *et al.*, 1985; Wells & Fersht, 1985, 1986; Bedouelle & Winter, 1986; Ho & Fersht, 1986; Jones *et al.*, 1986). Developments in the chemical synthesis of DNA fragments and recombinant DNA technology have enabled the modification of proteins by highly specific mutagenesis of their genes. Such structure-function relationships have been carried out for YTS from *B. stearothermophilus* (Wilkinson *et al.*, 1983). The crystal structure of YTS has been published (Bhat *et al.*, 1982), and that of WTS is proceeding (Coleman & Carter, 1984; Carter & Coleman, 1987). It is envisaged that the use of Procion dyes as affinity probes and labels will facilitate the identification of residues and domains within various substrate-binding sites of WTS and YTS. This may provide another starting point for site-directed mutagenesis experiments.

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