Probing the substrate-binding sites of aminoacyl-tRNA synthetases with the Procion dye Green HE-4BD

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A reactive bis-dichloro derivative of the Procion dye Green HE-4BD was shown to inactivate irreversibly methionyl-tRNA synthetase (MTS) from *Escherichia coli* and also tryptophyl-tRNA synthetase (WTS) and tyrosyl-tRNA synthetase (YTS) from *Bacillus stearothermophilus* at pH 8.5 and 37 °C. At a 5-fold excess of reactive dye over enzyme subunit concentration MTS was quantitatively inactivated within 20 min in the ATP/pyrophosphate exchange assay, whereas WTS and YTS show an 80 % loss of activity over the same time period. The inactivation is affected by the addition of substrates, which either protect (WTS and YTS) or promote (YTS with tyrosine) the dye-mediated enzyme inactivation. Green HE-4BD-OH was shown to be a competitive inhibitor of MTS with respect to MgATP, methionine and tRNA substrates.

INTRODUCTION

Structural studies on aminoacyl-tRNA synthetases (XTSs) have in the past generally provided more questions than answers. This family of enzymes play a central role in protein synthesis (Kisselev & Favorova, 1974), and the diversity of structure they display is intriguing, as judged from the viewpoint of their functional homology.

A wide variety of experimental approaches have been used to study the interactions between XTSs and their substrates, including comparison of sequence relationships between tRNAs aminoacylated by the same enzyme (Roe *et al.*, 1973), monitoring the fluorescence of specific bases of tRNA in interactions with its cognate synthetase (Ehrlich *et al.*, 1980) and studying the protection of phosphodiester bonds in yeast tRNA^{Val} by valyl-tRNA synthetase against alkylation by ethylnitrosourea (Vlassov *et al.*, 1981). The competitive labelling technique of Kaplan *et al.* (1971) involves comparing the reactivities of the ϵ -amino groups of lysine side chains of the protein towards an acylating agent in the free enzyme and enzyme-tRNA complex. This technique has been applied to tyrosyl-tRNA synthetase (YTS) by Bosshard *et al.* (1978) and to methionyl-tRNA synthetase (MTS) by Bruton (1979), and in both cases specific lysine residues with altered reactivities were identified. Recombinant DNA methods have also been used to produce mutant XTSs to identify the function of individual residues (Winter *et al.*, 1982). Active-site-directed inhibitors such as *p*-nitrophenyl-carbamoylaminoacyl-tRNA have proved useful in identifying important residues of MTS (Bruton & Hartley, 1970), which has also been inactivated by periodate-treated initiator tRNA (Hountondji *et al.*, 1979).

An alternative group of compounds, 'reactive triazine dyes', have been shown to make a useful contribution to affinity-labelling studies of this kind (Small *et al.*, 1982; Hughes *et al.*, 1984; Goward *et al.*, 1987). The triazine

dye Procion Brown MX-5BR has been shown to be a specific affinity label for tryptophyl-tRNA synthetase (WTS) (McArdell *et al.*, 1982, 1987; McArdell & Bruton, 1987).

In the affinity-chromatography study by Bruton & Atkinson (1979) it was demonstrated that several XTSs bind to a Green HE-4BD-Sepharose column. These authors suggested that the dye was occupying the tRNA-binding sites of these enzymes. Green HE-4BD is an aromatic polysulphonated dye, and results reported in the present paper lend support to the proposition that it can be used as a general tRNA-binding-site probe for XTSs.

MATERIALS AND METHODS

Materials

Samples of homogeneous tryptic fragment of L-methionyl-tRNA synthetase (MTS) from *Escherichia coli* (Hyafil *et al.*, 1976) (EC 6.1.1.10) as purified by Bruton *et al.* (1975) with specific activities of between 200 and 900 units/mg were gifts from Dr. C. J. Bruton (Parke-Davis Research Unit, Cambridge, U.K.). L-Tyrosyl-tRNA synthetase (YTS) from *Bacillus stearothermophilus* (EC 6.1.1.1) purified as described in Atkinson *et al.* (1979) and at a specific activity of 240 units/mg was also a gift from Dr. C. J. Bruton. Homogeneous L-tryptophyl-tRNA synthetase (WTS) from *B. stearothermophilus* (EC 6.1.1.2) was purified as described by Atkinson *et al.* (1979) to a specific activity of 1140 units/mg.

E. coli mixed tRNA species were prepared as described by Darbyshire *et al.* (1978).

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.). Na₄³²P₂O₇ and [³⁵S]methionine were purchased from Amersham International (Amersham, Bucks., U.K.). Procion Green HE-4BD was a gift from Dr. C. V. Stead

Abbreviations used: XTS, aminoacyl-tRNA synthetase; MTS, methionyl-tRNA synthetase; WTS, tryptophyl-tRNA synthetase; YTS, tyrosyl-tRNA synthetase. In these abbreviations the first letter designates the amino acid-specificity in the single-letter code.

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Fig. 1. Structures of (a) Green HE-4BD and (b) BD-Green 4BD

(ICI Organics Division, Blakeley, Manchester, U.K.); its structure is shown in Fig. 1(a).

Partially purified dye was prepared by repeated acid precipitation in 0.3 M-HCl followed by dissolving 5 g of dye in 50 ml of methanol and precipitation by the slow addition of diethyl ether. This procedure was repeated three times until the supernatant was clear. Dye purified in this way gave a single band in four different t.l.c. systems (McArdell, 1985).

A reactive bis-dichloro derivative of Green HE-4BD whose structure is shown in Fig. 1(b), and which was coded BD-Green 4BD, was kindly prepared and supplied by Dr. Stead.

Assay of XTS activities

XTS activity was measured by either the ATP/pyrophosphate-exchange assay or the tRNA-charging assay at 37 °C. In the ATP/pyrophosphate-exchange assay (Heinrikson & Hartley, 1967) the assay tubes contained, in a final volume of 200 µl, 20 µmol of Tris/HCl buffer, pH 8.0 or 8.5, 2 µmol of KF, 1 µmol of MgCl₂, 0.4 µmol of ATP, 0.4 µmol of Na₄³²P₂O₇ (specific radioactivity 80000–150000 c.p.m./µmol), 0.5 µmol of amino acid and 0.1 µmol of 2-mercaptoethanol. Bovine serum albumin (0.5 µmol) was normally included in assays of XTS activity, but was omitted in inhibitor studies as many dyes bind bovine serum albumin. Radioactive ATP generated in the assay was adsorbed on charcoal and its radioactivity counted. One unit of enzyme activity in this assay is defined as that amount that will exchange 1 µmol of pyrophosphate with ATP in 15 min at 37 °C.

MTS activity was alternatively measured by the tRNA-charging assay essentially as described by Heinrikson & Hartley (1967). The stock assay mixture contained in a final volume of 3 ml the following reagents: 0.8 mmol of Tris/HCl buffer, pH 7.7, 0.1 mmol of ATP, 0.45 mmol of MgCl₂, 0.8 µmol (5 µCi) of ³⁵S-labelled amino acid, 20 µmol of GSH and 1 mg of bovine serum albumin. Assay tubes contained 60 µl of stock assay mixture, 100 µl of *E. coli* tRNA (approx. 30 mg/ml) and 40 µl of enzyme to be assayed. One unit of enzyme activity in this assay is defined as that amount that will charge 1 nmol of tRNA with amino acid/min at 37 °C.

tRNA assay for specific tRNA species

Stock assay buffer contained, in a final volume of 10 ml, 6 mmol of Tris/HCl buffer, pH 7.5, 0.8 mmol of

MgCl₂, 2 mmol of NH₄Cl (most tRNA molecules are charged more efficiently in the presence of NH₄⁺ ions, although the reason for this is unknown), 0.16 mmol of ATP, 40 µmol of CTP and 196 µmol of 2-mercaptoethanol. A 50 µl portion of stock assay buffer, 20 µl of [³⁵S]methionine containing approx. 5 µCi/ml and 20 µl of unlabelled methionine (0.1 mM), 160 µl of water, 100 µl of crude tRNA to be assayed and 5 µl of homogeneous MTS (1 mg/ml) were mixed and incubated at 37 °C for 15 min. Tubes were chilled on ice after the addition of 4 ml of ice-cold 10% (w/v) trichloroacetic acid containing 2% (w/v) casamino acids (casein hydrolysate). After 30 min the precipitate was filtered off by using mild suction on Whatman GF/C filters, numbered with a graphite pencil. Filters were then washed with five 4 ml portions of ice-cold 10% trichloroacetic acid followed by 4 ml of ice-cold ethanol. Papers were then dried and their radioactivities counted in Aqualuma scintillant. Then [radioactivity (c.p.m.) in the assay]/[A₂₆₀ units of tRNA × specific radioactivity of methionine (c.p.m./pmol)] gives the pmol of amino acid charged/A₂₆₀ unit of tRNA. Pure tRNA of single specificity has a specific activity of 1500–1600 pmol/A₂₆₀ unit.

Protection studies

Inactivation studies were carried out in 400 µl total volume at 37 °C. MTS, WTS and YTS were each incubated with a 5-fold molar excess of dye, plus a 500-fold molar excess of either amino acid substrate, non-cognate amino acid or MgATP in 100 mM-Hepes buffer, pH 8.5, adjusted with NaOH. XTS was added at zero time and samples were taken and assayed for enzyme activity in duplicate at various intervals.

Hydroxylation of Green HE-4BD

Hydroxylated dye was prepared by incubating 1 mmol of purified dye, dissolved in water and adjusted to pH 10.5 with 5 M-NaOH, at 65 °C for 48 h. The pH was re-adjusted several times to keep it at pH 10.5, then after 48 h the pH was neutralized and the product was precipitated in 0.3 M-HCl and freeze-dried.

Determination of the stoichiometry of BD-Green 4BD binding to MTS

A 10 mg portion of the tryptic fragment of MTS (740 units/mg) was dialysed in N₂-gassed 20 mM-Hepes/NaOH buffer, pH 8.5, with three changes of buffer. The final concentration of the enzyme was 2.4 mg/ml. At

zero time a solution containing 1420 nmol of freshly prepared BD-Green 4BD was added to 142 nmol of the enzyme solution. Approx. 1 mg portions were taken at the intervals shown in Fig. 3 and rapidly mixed with a 100-fold excess of 2-mercaptoethanol over dye concentration. The stoichiometry of dye binding and ATP/pyrophosphate-exchange activity was determined after the labelled MTS had been separated from free dye by passage through a PD10 column (Pharmacia) equilibrated in water. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard, and the dye concentration from the absorption coefficient of the dye in 8M-urea (ϵ 57000 M⁻¹·cm⁻¹).

Determination of inhibitor constants

Inhibitor constants were determined in both the ATP/pyrophosphate-exchange and tRNA-charging assays. The inhibitor constant of hydroxylated Green HE-4BD (Green HE-4BD-OH) with respect to methionine was measured in 200 μ l total volumes of assay mix with MgATP (MgCl₂ and ATP in 2.5:1 ratio) as saturating fixed substrate (2 mM). The dye concentration was varied with a series of amino acid concentrations around the K_m for methionine.

The inhibitor constant of the dye with respect to MgATP was determined in assay mix of 400 μ l total

volume, with methionine 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 Na₄³²P₂O₇ (500000 c.p.m./ μ mol) and smaller amounts of enzyme were used in these experiments. This was in order to keep the percentage of total measured radioactivity in the assay below 10% of that available, as ATP is used and not regenerated.

Inhibitor constants were also measured by the tRNA-charging assay. The stock assay mix was used to dilute a series of methionine solutions to give final concentrations of 0.0167 mM, 0.033 mM, 0.05 mM, 0.0667 mM and 0.133 mM, each containing the same c.p.m. of [³⁵S]methionine/ml. Samples (90 μ l) of these assay mixes were each mixed with 150 μ l of tRNA and 60 μ l of MTS (0.1 nmol) for each assay, giving final methionine concentrations of 5 μ M, 10 μ M, 15 μ M, 20 μ M and 40 μ M.

RESULTS

Inactivation and protection studies

The effect of amino acid substrates and ATP on the inactivation of MTS, WTS and YTS by BD-Green 4BD in the ATP/pyrophosphate-exchange assay is shown in Figs. 2(a), 2(b) and 2(c). MTS is almost fully protected from inactivation by methionine; MgATP decreased the

Fig. 2. Protection of (a) MTS, (b) WTS and (c) YTS by ATP and amino acid substrates from inactivation by BD-Green 4BD

Inactivation conditions are described in the text. (a) ■, MTS plus dye; ●, MTS plus dye plus ATP; ○, MTS plus dye plus methionine; □, MTS plus dye plus glycine. (b) ■, WTS plus dye; □, WTS plus dye plus ATP; ○, WTS plus dye plus tryptophan; ●, WTS plus dye plus glycine. (c) ■, YTS plus dye; ●, YTS plus dye plus ATP; ○, YTS plus dye plus tyrosine; □, YTS plus dye plus glycine.

Fig. 3. Incorporation of BD-Green 4BD into MTS

Inactivation conditions are described in the text. ■, Covalent incorporation of BD-Green 4BD into MTS; ●, inactivation of ATP/pyrophosphate-exchange activity of MTS by BD-Green 4BD.

rate of inactivation of MTS but did not prevent the inactivation of at least 90 % MTS activity within 15 min. Conversely, WTS was significantly protected by MgATP, whereas the homologous amino acid tryptophan conferred only partial protection and glycine has little effect on the inactivation of WTS by BD-Green 4BD. The pattern of protection exhibited by the substrates of YTS was completely different. The homologous amino acid tyrosine increased the inactivation of YTS by BD-Green 4BD, whereas glycine and MgATP conferred a slight protection. Glycine was incorporated as a non-cognate amino acid to check dye inactivation by amines but had relatively little effect.

The apparent levelling off of the inactivation of YTS by BD-Green 4BD suggests that the k_p of the dye for this enzyme is higher than that for either MTS or WTS. The dye may also bind to other non-specific sites of YTS that do not affect the activity, thereby decreasing the apparent specific affinity as observed by rate of inactivation of YTS.

Fig. 3 shows the rapid incorporation of 1 mol of BD-Green 4BD/mol of MTS within 20 s. The concomitant loss of ATP/pyrophosphate-exchange activity is 40 %. After 15 min 4 mol of dye is incorporated/mol of enzyme with the loss of 90 % of the enzyme activity.

Inhibition studies

Fig. 4 shows the inhibition of MTS by Green HE-4BD-OH with respect to the methionine-binding site in the ATP/pyrophosphate-exchange assay. The K_m value for methionine was measured to be 42 μM . Green HE-4BD-OH was shown to have a strong competitive interaction with the methionine-binding site and displayed a K_i of 3 μM . Inhibition of MTS at the ATP-binding site by Green HE-4BD-OH was also shown to be competitive (Fig. 5). The K_m value for ATP was measured to be 200 μM and the K_i of Green HE-4BD-OH versus ATP was calculated to be 3 μM .

To begin to establish the kinetic parameters of Green HE-4BD-OH at the aminoacyl-tRNA^{Met} binding site the K_m values for methionine and tRNA^{Met} were measured by the tRNA-charging assay.

Multipoint rate assays with saturating tRNA^{Met} concentrations (100 μl of crude tRNA; approx. 100 mg/ml) were shown to be linear and appropriate radioactivity

counts were obtained with 0.25 nmol of MTS and a specific radioactivity of approx. 14000 c.p.m./nmol of [³⁵S]methionine per assay. The K_m for methionine in the tRNA-charging assay was calculated to be 20 μM and the K_m for tRNA^{Met} was 1 μM .

In order to calculate the inhibition constant of Green HE-4BD-OH versus methionine in the tRNA-charging assay, the procedure used to calculate the K_m for methionine was repeated with Green HE-4BD-OH included in the assays at concentrations between 5 μM and 300 μM . Fig. 6(a) shows the rates of aminoacylation of tRNA^{Met} at methionine concentrations of 10 μM , 15 μM , 20 μM and 40 μM , all incubation mixtures containing 30 μM -Green HE-4BD-OH. The inhibition of

Fig. 4. Inhibition of MTS by Green HE-4BD-OH at the methionine-binding site

●, No dye; ◆, 2 μM -dye; ■, 10 μM -dye. Assays were carried out as described in the text with 1.5 pmol of MTS per assay and $\text{Na}_4^{32}\text{P}_2\text{O}_7$ at a specific radioactivity of 42000 c.p.m./ μmol .

Fig. 5. Inhibition of MTS by Green HE-4BD-OH at the ATP-binding site

●, No dye; ■, 10 μ M-dye; ▲, 30 μ M-dye. Assays were carried out as described in the text with 1.2 pmol of MTS per assay and $\text{Na}_2^{32}\text{P}_4\text{O}_7$ at a specific radioactivity of 500 000 c.p.m./ μ mol.

MTS by Green HE-4BD-OH was shown to be comparative (Fig. 6b) at the methionine-binding site with a K_i value of 34 μ M.

The inhibition constant of Green HE-4BD-OH versus tRNA^{Met} was competitive (Fig. 7) and was calculated to

Fig. 7. Inhibition of MTS at the tRNA^{Met} -binding site by Green HE-4BD-OH

●, No dye; ○, 30 μ M-dye; ■, 100 μ M-dye. [^{35}S]Methionine was at a fixed saturating concentration of 135 μ M. Crude *E. coli* tRNA was included to give final concentrations of tRNA^{Met} in the assay between 0.1 μ M and 2.6 μ M.

be 28 μ M, similar to the value of 34 μ M calculated for the interaction at the methionine-binding site.

DISCUSSION

The principal tRNA-binding site for most aminoacyl-tRNA synthetases is proposed to be along the side of the tRNA molecule that has a constant number of nucleotides in all species (Rich, 1974). This portion of the tRNA molecule spans from the anticodon to the 3'

Fig. 6. (a) Rates of tRNA-charging activity of MTS at various methionine concentrations, and (b) Dixon plot of the inhibition of MTS by Green HE-4BD-OH at the methionine-binding site in the tRNA-charging assay

(a) Rates of tRNA-charging activity of MTS at various methionine concentrations. All assays shown contained Green HE-4BD-OH at a final concentration of 30 μ M. Methionine concentrations in the assay were: ●, 10 μ M; ○, 15 μ M; ▲, 20 μ M; △, 40 μ M. The [^{35}S]methionine assay mix contained 366×10^5 c.p.m./ml. This corresponds to the following specific radioactivities: 1109 c.p.m./pmol for 10 μ M-methionine, 739 c.p.m./pmol for 15 μ M-methionine, 554 c.p.m./pmol for 20 μ M-methionine and 277 c.p.m./pmol for 40 μ M-methionine. Assays were carried out with 0.25 nmol of MTS per assay and 100 μ l of crude *E. coli* tRNA containing 2.13 mg of tRNA^{Met} /ml (giving a final concentration of 42.6 μ M- tRNA^{Met}). (b) Dixon plot of the inhibition of MTS by Green HE-4BD-OH at the methionine-binding site in the tRNA-charging assay. The rates of MTS activity were calculated by linear-regression analysis of the slopes obtained at the following concentrations of methionine: □, 10 μ M; ■, 15 μ M; ○, 20 μ M; ●, 40 μ M.

terminus, which is a distance of approx. 7.6 nm. Depending on the particular enzyme and tRNA, the synthetase may encompass the entire area or only a portion of it. Green HE-4BD is approx. 4.0 nm in length and therefore could bind across a significant part of the tRNA-binding site.

The competitive kinetics shown by the binding of Green HE-4BD-OH versus tRNA^{Met}, methionine and MgATP with MTS demonstrates the direct competition between the binding of substrates and inhibitor. This suggests (1) that the dye is large enough to occlude all three binding sites simultaneously, which must therefore be spatially very close, or (2) that the binding of the dye to one site causes a conformational change that prevents the binding of the other substrates, or (3) that the dye binds specifically to more than one site.

The fact that methionine fully protects MTS suggests there is one dye-binding site, which occludes the entire active site, and that the substrate-binding sites are concomitantly very close together, or the conformational change that occurs on binding methionine (Zelwer *et al.*, 1982) excludes the dye completely from MTS. MgATP lowered the rate of enzyme inactivation, which suggests that this binding decreases the affinity of the dye for MTS.

The discrepancy between the K_i for Green HE-4BD-OH versus methionine in the ATP/pyrophosphate-exchange assay and tRNA-charging assay, 3 μ M and 34 μ M respectively, can be explained by an interrelation between tRNA and amino acid-activating sites; this interrelationship has been previously reported by Jacques & Blanquet (1977). In the crystal structure of MTS (Zelwer *et al.*, 1982) the substrate-binding sites are not clearly defined. MTS crystals crack on diffusion with L-methionine, and attempts to achieve co-crystallization in an MTS/methionine complex were unsuccessful. One characteristic feature of the structure of MTS is the presence of a large depression located on one side of the molecule. Neutron-scattering data (Dessen *et al.*, 1978) have indicated that the tRNA centre of mass should lie very close to the centre of mass of the native enzyme. The tRNA (or dye) molecule could therefore occupy the interspatial area between the two main domains of MTS. The long dye molecule could also protrude into, interact with or cause a conformational change at the methionine- and MgATP-binding domains.

The initial rapid incorporation of 1 mol of dye/mol of MTS subunit is indicative of a specific incorporation at the active site. More dye becomes covalently attached to the enzyme at less-specific sites as the incubation proceeds owing to the extreme reactivity of the bis-dichloro dye.

It is hoped that the reactive dye BD-Green-4BD can be used as an effective probe with which to identify reactive residues within the tRNA^{Met}- and methionyladenylate-binding site of MTS.

Both MTS and YTS have been shown to possess a nucleotide-binding fold (Risler *et al.*, 1981; Blow *et al.*, 1983). Early studies with the Procion dye Cibacron Blue F3G-A showed that it interacted specifically, but not exclusively, with nucleotide-requiring enzymes at the dinucleotide fold (Stellwagen, 1977; Beillman *et al.*, 1979; Edwards & Woody, 1979). It is tempting to predict that members of the XTS family may all possess a nucleotide fold and that Green HE-4BD may specifically, but not exclusively, interact with this structure.

WTS and YTS were specifically protected from

inactivation by BD-Green 4BD by substrates, although an interesting enhancement of inactivation occurred with tyrosine and YTS. This suggests that on binding tyrosine YTS undergoes a conformational change that opens up the dye-binding site. It is possible that a basic difference in enzyme mechanism exists in this case, and further studies may shed light on this question.

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