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AFFINITY CHROMATOGRAPHY ON IMMOBILISED TRIAZINE DYES

STUDIES ON THE INTERACTION WITH MULTINUCLEOTIDE-DEPENDENT ENZYMES

YANNIS D CLONIS and CHRISTOPHER R LOWE

Department of Biochemistry, University of Southampton, Bassett Crescent East, Southampton, SO9 3TU, (U K)

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Summary

A systematic investigation into the interaction of several triazinyl dyes with two enzymes from purine metabolism, IMP dehydrogenase (IMP NAD^+ oxidoreductase, EC 1.2 1.14) and adenylosuccinate synthetase (IMP L-aspartate ligase (GDP-forming), EC 6.3.4.4) has been conducted. Evidence from kinetic inhibition studies, enzyme inactivation with specific affinity labels and specific elution techniques from agarose-immobilised dyes indicate that triazine dyes such as Procion Blue H-B (Cibacron Blue F3G-A), Red HE-3B and Red H-3B are able to differentiate between the nucleotide-binding sites of these enzymes. This information has been exploited to design specific elution techniques for the purification of these enzymes by affinity chromatography

Introduction

It is well established that the anthraquinone dye Procion Blue H-B or Cibacron Blue F3G-A when covalently bound to agarose is an exceedingly useful 'group-specific' adsorbent for the purification of a plethora of proteins by affinity chromatography [1—6]. For example, immobilised Cibacron Blue F3G-A appears to be a particularly effective adsorbent for the purification of pyridine nucleotide-dependent dehydrogenases, kinases, flavoproteins, glycolytic enzymes, blood proteins and a number of seemingly unrelated proteins [5,6]. Several studies have been initiated to establish the basis for these selec-

Abbreviations XMP, xanthosine 5′-monophosphate, Cl^6-IMP, 6-chloro-inosine 5′-monophosphate

tive interactions [7—10] and it has been tentatively concluded that the polysulphonated aromatic chromophore of the dye mimics, at least in part, the naturally occurring biological heterocycles [9]. However, more recent physicochemical studies suggest that the anthraquinone dyes are not highly specific analogues of nucleotide mono-, di and triphosphates, NAD^+, $NADP^+$, flavins, acetyl-CoA or folic acid since, in fact only part of the chromophore accurately reflects coenzyme binding to the complementary proteins [7—10,11] Not surprisingly, therefore, a number of other triazinyl dyes have been tested as potential replacements for, or complements to, Cibacron Blue F3G-A in the hope of achieving either different or improved specificity [5,12—14]. In particular, the reactive azo dye Procion Red HE-3B has proven a useful addition to Cibacron Blue F3G-A in that it displays enhanced specificity and affinity for $NADP^+$-dependent dehydrogenases [5,15] as well as affinity for proteins such as carboxypeptidase G1 [12] and a number of NAD^+-dependent enzymes [14,16]. It thus appears that immobilised Procion Red HE-3B behaves as a 'group-specific' affinity ligand similar to Cibacron Blue F3G-A but with different selectivity and binding properties.

Few studies have been conducted to establish at which site on the enzymes these dyes actually bind. This information is crucial for the optimalisation of appropriate adsorption and specific elution conditions for affinity chromatography. Where studies have been performed, primarily with Cibacron Blue F3G-A, a rather confusing medley of strictly competitive, non-competitive, mixed or other unusual types of enzyme inhibition have been observed [17]. To the author's knowledge no studies have been performed to establish whether triazine dyes can differentiate between structurally related binding sites such as those found in proteins binding several different purine nucleotides. This paper describes an investigation into the interaction between Cibacron Blue F3G-A and Procion Red HE-3B and two multiple-nucleotide binding enzymes from purine metabolism, inosine 5′-monophosphate dehydrogenase (IMP NAD^+ oxidoreductase, EC 1.2 1.14) and adenylosuccinate synthetase (IMP. L-aspartate ligase (GDP-forming) EC 6 3.4.4). These studies demonstrate unequivocally that the chromatographic behaviour of these enzymes on immobilised triazine dyes can be attributed to the ability of triazine dyes to discriminate between nucleotide binding sites on the same enzyme.

Materials and Methods

Materials NAD^+, GMP (disodium salt) and L-aspartate were obtained from British Drug Houses, Poole, Dorset, U.K. Inosine 5′-monophosphate (IMP), adenosine 5′-monophosphate (AMP), xanthosine 5′-monophosphate (XMP), GTP (disodium salt, type I) and reduced glutathione were from the Sigma (London) Chemical Co., whilst DEAE-cellulose was from Whatman Biochemicals, Maidstone, Kent. Sepharose 4B was purchased from Pharmacia (G.B.) Ltd. while pyruvate kinase (rabbit muscle, 200 units/mg) and phospho*enol*pyruvate (tricyclohexylammonium salt) were from the Boehringer Corporation, Lewes, Sussex, U.K. Cibacron Blue F3G-A was a gift from Ciba-Geigy (U.K.), Manchester, whilst the other Procion dyes, including Procion Red HE-3B, were

a generous and much appreciated gift from Dr. C.V. Stead, Organics Division, ICI, Manchester, U.K. The active site label, 6-chloro-inosine 5′-monophosphate (Cl^6-IMP) was from Calbiochem-Behring Corp.

Bacterial strains and preparation of cell-free extracts *Escherichia coli* K12 strain W3110 was used as the prototrophic parental strain for the isolation of *gua* and *Pur* mutants. Stock cultures were maintained on Dorest egg slopes [18]. Cell-free extracts of the mutants PL1068 (*guaA* 48) and MW1068 (*PurB* 773) were prepared as described previously [19].

Protein assays The protein concentration in crude cell-free extracts and in column effluents was determined by the ultraviolet absorption method of Warburg and Christian [20] The protein concentration of purified preparations of IMP dehydrogenase and adenylosuccinate synthetase was determined by the method of Lowry et al. [21] using crystalline bovine serum albumin (fraction V) as standard.

Enzyme assays. The reaction mixture for the assay of IMP dehydrogenase contained the following in a total volume of 1 ml: Tris-HCl, pH 7.5, 50 μmol, KCl, 50 μmol, GSH, 5.0 μmol, NAD^+, 1.25 μmol; IMP, 1.35 μmol, enzyme, 0—0.030 units [18,19]. The reaction was initiated by the addition of enzyme to the cuvette (10 mm pathlength) and followed continuously at 35°C in a Unicam SP1800 spectrophotometer by measuring the production of NADH at 340 nm. The reference cuvette contained all components of the assay mixture except IMP. 1 unit of enzyme activity is defined as the amount of enzyme required to transform 1 μmol of substrate/min at 35°C using a molar extinction coefficient of $6.225 \cdot 10^3$ l · mol^{-1} · cm^{-1} for NADH at 340 nm.

The reaction mixture for the assay of adenylosuccinate synthetase contained the following in a total volume of 1 ml: Hepes-KOH, pH 7.0, 50 μmol, $MgCl_2$, 5 μmol, L-aspartate, 5 μmol, IMP, 0.65 μmol, phospho*enol*pyruvate, 0.5 μmol, GTP, 0.25 μmol; pyruvate kinase, rabbit muscle, 10 units; adenylosuccinate synthetase, 0—0.8 units. 1 unit of enzyme activity is defined as the amount of enzyme required to produce 1 nmol product/min at 25°C using a molar extinction coefficient of $11.7 \cdot 10^3$ l · mol^{-1} · cm^{-1} for adenylosuccinate at 290 nm [19].

Inactivation of IMP dehydrogenase with Cl^6-IMP and the effect of triazine dyes IMP dehydrogenase was inactivated with Cl^6-IMP at 25°C in a 1 ml total reaction mixture containing Tris-HCl, pH 7.5, 50 μmol, KCl, 50 μmol, enzyme, 0.1 unit and Cl^6-IMP, 1.37 nmol. Samples (10 μl) were withdrawn for assay of IMP dehydrogenase activity over a period of 1 h. The control reaction mixture contained all components except Cl^6-IMP (1.37 nmol). The inactivation by Cl^6-IMP was also performed in the presence of IMP (1 μmol), Cibacron Blue F3G-A (1 μmol) and Procion Red HE-3B (1 μmol) respectively. IMP dehydrogenase was also inactivated by the two dyes, Cibacron Blue F3G-A and Procion Red HE-3B, at 25°C in a reaction mixture (1 ml total volume) containing Tris-HCl, pH 7.5, 50 μmol; KCl, 50 μmol; enzyme, 0.1 unit and triazine dye, 1 μmol. The concentrations of the nucleotides and dyes were determined spectrophotometrically at λ_{max} using the following molar extinction coefficients (l · mol^{-1} · cm^{-1}): IMP (250 nm; 12 300) [19] Cibacron Blue F3G-A (620 nm; 13 300) [9] and Procion Red HE-3B (530 nm, 30 100). The structures of the two triazine dyes, Cibacron Blue F3G-A and Procion Red

HE-3B are described in Ref. 29. Procion Blue H-B is identical to Cibacron Blue F3G-A.

Kinetic inhibition studies with triazine dyes Initial velocities for the IMP dehydrogenase-catalysed reaction with IMP as variable substrate were measured in 1 ml total volume at 25°C containing. Tris-HCl, pH 7.5, 50 μmol; KCl, 50 μmol; NAD^+, 1.25 μmol; GSH, 5 μmol; enzyme, 0.0148 units (at 25°C) and IMP, 3.675—98.0 nmol, in the absence of and in the presence of 5.9 and 17.7 nmol Cibacron Blue F3G-A and 30 and 50 nmol Procion Red HE-3B. With NAD^+ as variable substrate, the reaction cuvette contained in a total volume of 1 ml· Tris-HCl, pH 7.5, 50 μmol; KCl, 50 μmol; GSH, 5 μmol; IMP, 1.35 μmol: enzyme, 0.0148 units (at 25°C) and NAD^+, 42.95—601.3 nmol in the absence and in the presence of 17.7 and 29.5 nmol Cibacron Blue F3G-A and 30 and 50 nmol Procion Red HE-3B.

Initial velocities for the adenylosuccinate synthetase catalysed reaction with IMP as variable substrate were measured at 25°C in 1 ml total volume containing: Hepes-KOH, 50 μmol; $MgCl_2$, 5 μmol; L-aspartate, 5 μmol, GTP, 0.25 μmol; phospho*enol*pyruvate, 0.5 μmol; pyruvate kinase, 10 units; adenylosuccinate synthetase, 1 unit; IMP, 3.65—109.5 nmol in the absence of dye and in the presence of 1.2 nmol Cibacron Blue F3G-A or 5 nmol Procion Red H-3B, respectively. With GTP as variable substrate, initial velocities were measured in the absence of dye and in the presence of 1.2 nmol Cibacron Blue F3G-A or 10 nmol Procion Red H-3B in the above reaction mixture containing: IMP, 0.65 μmol and GTP, 4—40 nmol. With L-aspartate as variable substrate the reaction mixture contained· IMP, 0.65 μmol, GTP, 0.25 μmol and L-aspartate, 100—2000 nmol.

Kinetic constants were deduced from weighted linear regression analysis of double reciprocal plots using a Hewlett-Packard 9810 A Programmable calculator.

The IMP dehydrogenase used in these studies was a homogenous preparation of specific activity 9.1 units/mg prepared by a previously published procedure [19]. The adenylosuccinate synthetase was a partially purified preparation of specific activity 508 units/mg (Stage 4, Table V). The concentrations of the nucleotides and dyes were determined spectrophotometrically: GTP (λ_{max} 253 nm; ϵ_M 13 700 $l \cdot mol^{-1} \cdot cm^{-1}$); NAD^+ (λ_{max} 260 nm, ϵ_M 17 800 $l \cdot mol^{-1} \cdot cm^{-1}$) [19]; Procion Red H-3B (λ_{max} 530 nm; ϵ_M 18 100 $l \cdot mol^{-1} \cdot cm^{-1}$).

Dye immobilisation The monochlorotriazine dyes, Cibacron Blue F3G-A and Procion Red HE-3B were covalently attached to Sepharose 4B essentially according to Lowe et al. [14]. To 10 g exhaustively washed (2 l distilled water) Sepharose 4B was added 100 mg dye in water (10 ml) followed by NaCl (2 ml; 22% w/v) to give a final concentration of 2% (w/v) NaCl. The gel was slowly tumbled at room temperature (25°C) for 30 min prior to adding solid Na_2CO_3 to a final concentration of 1% (w/v). The gels were tumbled gently for 5 days at 25°C and then washed exhaustively with water and 1 M KCl until no dyestuff was evident in the washings. The gels were stored moist at 0—4°C.

Immobilised dye concentrations were determined by acid hydrolysis of the gels [4]. Moist gel (30 mg) was transferred to 5 M HCl (0.6 ml) incubated at 37°C for 5 min and 2.5 M sodium phosphate buffer, pH 7.5, (2.4 ml) added. Dye concentrations were determined spectrophotometrically at λ_{max} using the

molar extinction coefficients quoted above.

Chromatographic procedures. Analytical scale chromatography of cell-free extracts of *E coli* mutants was performed as follows. Dialysed cell-free extracts of the *guaA* mutant PL1068 (100 µl, 0.6 units IMP dehydrogenase) or the *purB* mutant MW1068 (200 µl; 6.5 units adenylosuccinate synthetase) were applied to columns (0.45 × 3.2 cm) containing agarose-immobilised dyes (0.5 g moist weight gel). Unbound protein was washed off with starting buffer (10—12 ml) and enzyme activity eluted with a linear gradient of IMP (0—20 mM, 20 ml total volume, adenylosuccinate synthetase) or with linear gradients of various nucleotides (20 ml total volume, IMP dehydrogenase; see Results for details). Fractions (1.5 ml) were collected at a flow rate of 7.5 ml/h and assayed for enzyme activity, protein and eluant. The irrigating buffer for IMP dehydrogenase chromatography was 50 mM potassium phosphate, pH 7.0, and for adenylosuccinate synthetase, 50 mM Tris-HCl, pH 7.5, containing 1.5 mM $MgCl_2$ and 1 mM dithiothreitol. The concentration of the eluting nucleotides were determined spectrophotometrically at λ_{max} using the following molar extinction coefficients ($l \cdot mol^{-1} \cdot cm^{-1}$): AMP (260 nm, 15 300), GMP (252 nm, 13 700) and XMP (280 nm, 8900)

Large scale purification of E coli adenylosuccinate synthetase. Two overnight cultures (each 5 ml) of *purB* mutant MW1068 in minimal medium containing glucose (2 mg/ml) and supplemented with adenine (20 µg/ml) and thiamine hydrochloride (vitamin B-1, 4 µg/ml) were transferred to minimal medium (2 × 200 ml) containing glucose (2 mg/ml) and supplemented with adenine (10 µg/ml) and vitamin B-1 (4 µg/ml). The cultures were shaken for 8 h at 37°C, after which two samples (each 200 ml) were transferred to two aspirators, each containing 20 l of minimal medium containing glucose (2 mg/ml) and supplemented with adenine (5 µg/ml), vitamin B-1 (4 µg/ml) and casein hydrolysate (1 mg/ml). The cultures were incubated at 37°C for 12—15 h under stirring and continuous aeration with filtered air at 15 l/min. The cells were harvested in a flow-through Sharples centrifuge at 50 000 × *g*. All subsequent operations were performed at 0—4°C. The cell paste was washed with 50 mM Tris-HCl buffer, pH 8.0, containing 1.5 mM $MgCl_2$ and 2 mM dithiothreitol (approx. 180 ml), spun down at 19 000 × *g* (MSE centrifuge, 6 × 250 ml head) for 15 min and resuspended in 30 ml of the same buffer. The cells were sonicated at 20—21 kHz (6.5 peak-to-peak amplitude) in six bursts of 2 min leaving 1 min intervals between bursts in a 100 W MSE ultrasonic disintegrator. Cell debris was removed by centrifugation at 30 000 × *g* for 45 min. The supernatant was dialysed overnight against 20 vol. of 50 mM Tris-HCl buffer, pH 8.0, containing 1.5 mM $MgCl_2$ and 2.0 mM dithiothreitol.

The extract (50 ml, 5290 units of adenylosuccinate synthetase; 2.38 g protein) was applied to a column (3.4 × 14.5 cm, 130 g moist weight) of DEAE-cellulose (DE-52, Whatman, U.K.) equilibrated with the same buffer as above. Non-adsorbed protein was washed off with irrigating buffer (216 ml) and adenylosuccinate synthetase activity eluted with a linear gradient of NaCl (0—0.4 M, 1.5 l total volume). Fractions (18 ml) were collected at a flow rate of 200 ml/h and those containing adenylosuccinate synthetase activity were pooled and taken to 50% saturation with solid $(NH_4)_2SO_4$ over a 15 min period under stirring. The mixture was left for a further 15 min and the precipitated

protein collected by centrifugation at 30 000 × *g* for 30 min. The supernatant was taken to 80% saturation with solid $(NH_4)_2SO_4$ over a 15 min period under stirring, the mixture left for a further 15 min and the precipitated protein collected by centrifugation at 30 000 × *g* for 30 min. The pellet was dissolved in 2.7 ml 50 mM Tris-HCl buffer, pH 7.5, containing 1.5 mM $MgCl_2$ and 2.0 mM dithiothreitol and dialysed against 2 l of the same buffer over a 6 h period.

The dialysed adenylosuccinate synthetase preparation (7.1 ml; 4786 units adenylosuccinate synthetase; 284 mg protein) was applied to a column (1.1 × 16.5 cm, 16 g moist weight gel) of agarose-bound Procion Red H-3B (1.4 μmol dye/g moist gel) equilibrated with the same buffer as above. Flow was interrupted for 5 min, whence non-adsorbed protein was subsequently washed off with starting buffer (90 ml) and adenylosuccinate synthetase activity eluted with a linear gradient of KCl (0—1 M, 120 ml total volume). Fractions (9 ml) were collected at a flow rate of 30 ml/h and those containing adenylosuccinate synthetase activity pooled and concentrated (22 lb/inch^2) to 2 ml in an AMICON concentrator supplemented with a ULVAC ultrafiltration membrane (10 000 cut-off; ChemLab Instruments Ltd.). The concentrate was then dialysed overnight against 1 l of the same buffer.

The dialysed preparation (2.8 ml, 2128 units adenylosuccinate synthetase; 16.8 mg protein) was applied to a column (1.1 × 13.5 cm, 12.8 g moist weight gel) of agarose-bound Procion Blue H-B (0.8 μmol dye/g moist gel) equilibrated with 50 mM Tris-HCl buffer, pH 7.5, containing 1.5 mM $MgCl_2$ and 2.0 mM dithiothreitol. Flow was interrupted for 5 min, whence non-adsorbed protein was washed off with irrigating buffer (66 ml) and adenylosuccinate synthetase activity eluted with an eluant (50 ml) comprising IMP (4 mM), GTP (2 mM) and L-aspartate (10 mM) in the above irrigating buffer. Fractions (9.5 ml) were collected at a flow rate of 40 ml/h and those containing adenylosuccinate synthetase activity pooled and concentrated (22 lb/inch^2) to 1.8 ml (1829 units of adenylosuccinate synthetase; 3.6 mg total protein, 508 units/mg) in an AMICON concentrator containing a ULVAC ultrafiltration membrane (10 000 cut-off).

Results and Discussion

The interaction of E coli IMP dehydrogenase with triazinyl dyes

E coli inosine 5′-monophosphate dehydrogenase catalyses the interconversion of IMP and XMP with the concomitant reduction of NAD^+ to NADH and can be purified by affinity chromatography on immobilised AMP and IMP [18,19], GMP [23] and a number of chemically distinct triazinyl dyes [14]. Under appropriate conditions the enzyme is adsorbed to both agarose-immobilised Cibacron Blue F3G-A (Procion Blue H-B) and Procion Red HE-3B [14]. It is therefore of interest to establish whether this enzyme binds to these adsorbents though its substrate/product site, i.e. IMP/XMP, its coenzyme site, i.e. NAD^+/ NADH, or neither of these sites. Fig. 1(a) illustrates the inhibitory effect of free Cibacron Blue F3G-A (Procion Blue H-B) on the initial velocity of the IMP dehydrogenase reaction at constant saturating NAD^+ concentration and variable IMP concentration. Cibacron Blue F3G-A appears to be a strictly competitive inhibitor with respect to IMP with a competitive inhibition con-

stant, K_i = 5.7 μM. On the other hand, under similar conditions, free Procion Red HE-3B displays mixed inhibition with respect to IMP (Fig. 1(b). However, with IMP as fixed substrate and NAD^+ as variable substrate, Procion Red HE-3B displays weak competitive inhibition versus NAD^+ with K_i = 114.3 μM, as illustrated in Fig. 2(b). Under the same conditions, Cibacron Blue F3G-A displays mixed inhibition versus NAD^+ as variable substrate (Fig. 2(a)). It seems likely therefore that Cibacron Blue F3G-A binds preferentially to the substrate (IMP) site whilst Procion Red HE-3B interacts preferentially with the coenzyme (NAD^+) site. This tentative conclusion is supported by the data shown in Fig. 3 which illustrates the inactivation of IMP dehydrogenase with the substrate site-specific affinity label, Cl^6-IMP [22,33]. Under the conditions cited, Cl^6-IMP rapidly inactivated IMP dehydrogenase in such a manner that specific protection is afforded by IMP. Similar protection against inactivation by Cl^6-IMP is afforded by Cibacron Blue F3G-A but no protection is observed with Procion Red HE-3B. Preferential binding of Cibacron Blue F3G-A and Procion Red HE-3B, respectively, to the IMP and NAD^+ binding sites of IMP dehydrogenase is further substantiated by the specific elution of *E coli* IMP dehydrogenase from immobilised dye columns. Table I illustrates that under approximately equivalent immobilised ligand concentrations, 0.8—1.0 μmol dye/g moist weight gel, IMP dehydrogenase may be biospecifically eluted from an immobilised Procion Red HE-3B column with AMP and NAD^+ with recoveries of enzyme activity of 60 and 50%, respectively, but not with IMP or its competitive nucleotides GMP and XMP [18]. On the other hand, IMP is an especially effective eluant of the enzyme, with quantitative recovery of enzyme activity, from agarose-immobilised Cibacron Blue F3G-A. Other nucleotides, such as AMP, NAD^+, GMP and XMP, also proved effective in eluting the enzyme from this adsorbent although with lower yields of recovered enzyme activity (68—78%). The ability of AMP and NAD^+ to act as eluants may indicate some binding of Cibacron Blue F3G-A to the NAD^+-site as has been found for a number of other dehydrogenases [5,17].

The interaction of E coli adenylosuccinate synthetase with immobilised triazinyl dyes

Adenylosuccinate synthetase (EC 6.3.4.4) catalyses the GTP-dependent synthesis of adenylosuccinate from IMP and L-aspartate [19]. Table II illustrates the chromatography of the *E coli* enzyme on a number of immobilised Procion dyes at room temperature. Preliminary studies indicated that on chromatography of a crude bacterial extract on small columns of immobilised Procion Green HE-4BD, Procion Yellow MX-R and Cibacron Blue F3G-A at 0—4°C and at ambient temperature, 23—24°C, consistently higher recoveries of enzyme activity (20—60%) were obtained at the higher temperature. All subsequent chromatographic steps were therefore performed at room temperature [24]. Examination of Table II reveals that, despite disparities in immobilised dye concentrations, *E coli* adenylosuccinate synthetase appears to display greater affinity for the green, red and blue dyes than the yellow and orange dyes. Thus, for example, at comparable ligand concentrations of 1.0—1.1 μmol/g moist weight gel, the enzyme displays no affinity for immobilised Procion Yellow H-A but binds tightly to immobilised Procion Red HE-3B and Cibacron Blue

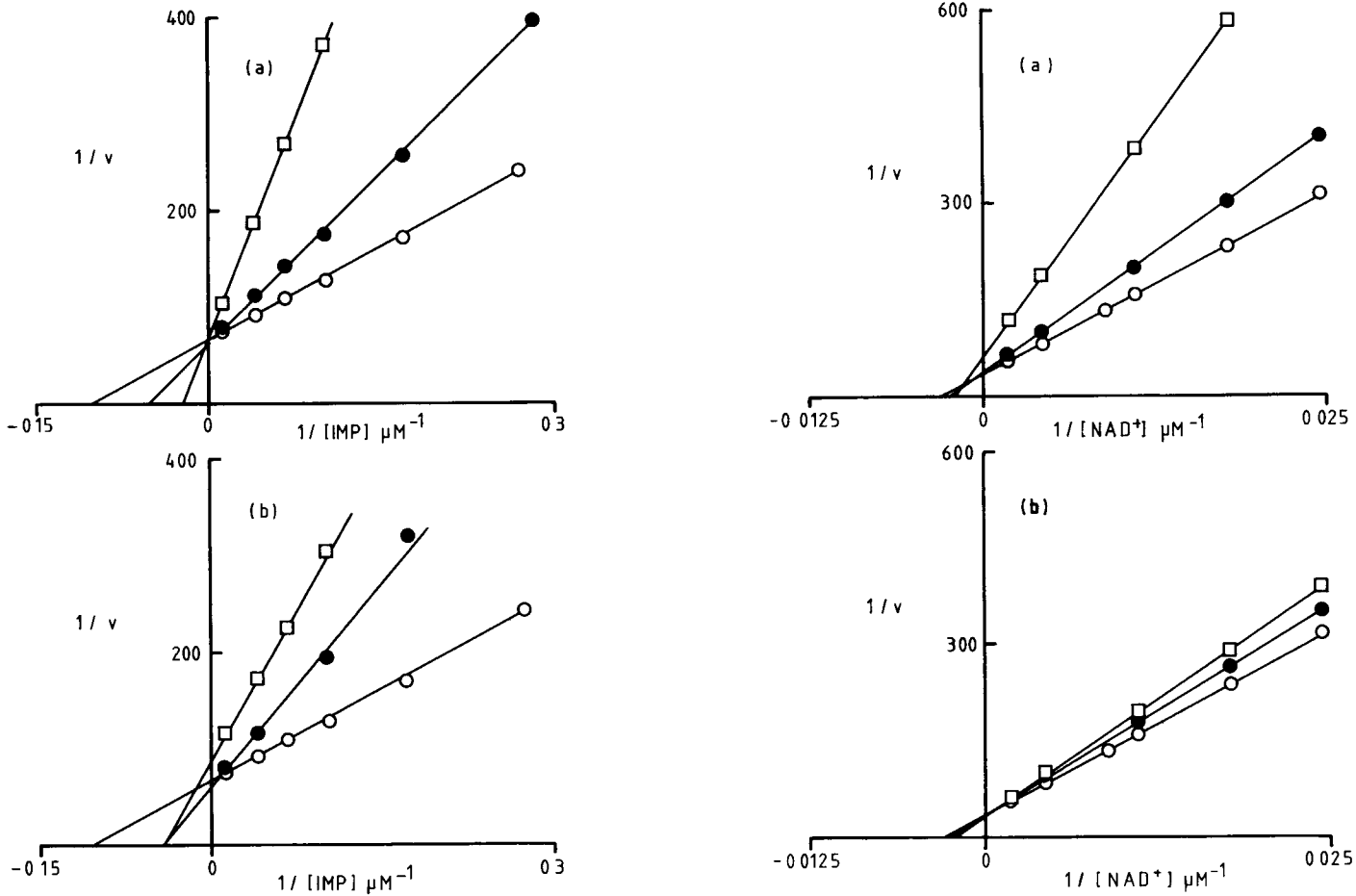

Fig 1 Inhibition of *E coli* IMP dehydrogenase by Procion Blue H-B and Procion Red HE-3B at different IMP concentrations The reaction mixture contained in 1 ml total volume at 25°C IMP, 3 7—98 0 nmol, NAD^+, 1 25 mol, Tris-HCl buffer, pH 7 5, 50 μmol, KCl, 50 μmol, GSH, 5 μmol, enzyme, 10 μl, 0 0148 units Initial velocity (v) is quoted in μmol NADH produced/min Enzyme assayed in the absence of dye (○) or in the presence of (a) 5 9 nmol Procion Blue H-B (●), 17.7 nmol Procion Blue H-B (□), (b) 30 nmol Procion Red HE-3B (●), 50 nmol Procion Red HE-3B (□)

Fig 2 Inhibition of *E coli* IMP dehydrogenase by Procion Blue H-B and Procion Red HE-3B at different NAD^+ concentrations The reaction mixture contained in 1 ml total volume at 25°C NAD^+, 42 9—601 3 nmol, IMP, 1 35 μmol, Tris-HCl buffer pH 7 5, 50 μmol, KCl, 50 μmol, GSH, 5 μmol, enzyme, 10 μl, 0 0148 units Initial velocity (v) is quoted in μmol NADH produced/min Enzyme assayed in the absence of dye (○) or in the presence of (a) 17 7 nmol Procion Blue H-B (●), 29 5 nmol Procion Blue H-B (□), (b) 30 nmol Procion Red HE-3B (●), 50 nmol Procion Red HE-3B (□)

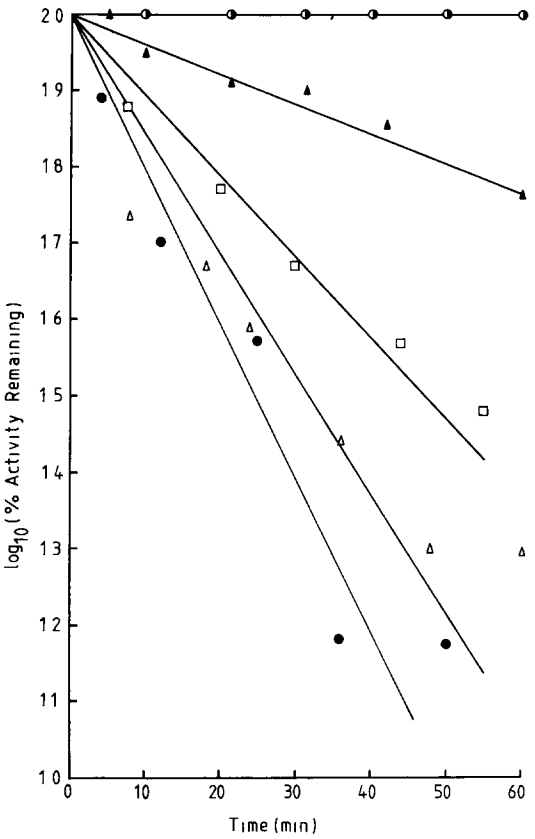

Fig 3 Inactivation of *E coli* IMP dehydrogenase by Cl^6-IMP in the presence of Procion Blue H-B or Procion Red HE-3B IMP dehydrogenase (0 1 unit, 10 µl) was incubated at 25°C with Cl^6-IMP (1 37 nmol) in 50 mM potassium phosphate buffer pH 7.0 containing 50 mM KCl in 1 ml total incubation volume with the following additions IMP, 1 µmol (▲), Procion Blue H-B, 1 µmol (□), Procion Red HE-3B, 1 µmol (●), none (△), control incubation (◑) containing no Cl^6-IMP Samples (10 µl) were removed at the time intervals indicated and assayed for enzyme activity as described in the text

TABLE I

SPECIFIC ELUTION OF *E COLI* IMP DEHYDROGENASE FROM IMMOBILISED TRIAZINE DYES

Chromatography and enzyme assays were performed as described in the text Elution was effected with linear gradients (20 ml total volume) of AMP and NAD^+ (0—20 mm), IMP (0—50 mM) and GMP and XMP (0—40 mM) Figures refer to the concentration of eluant (mM) required to elute maximal activity of enzyme on a linear gradient and figures in parenthesis refer to the % of applied enzyme activity (0 6 units) bound and subsequently eluted Immobilised dye concentrations 1 0 µmol Procion Red HE-3B/g moist gel, 0 8 µmol Procion Blue H-B/g moist gel No elution could effected with IMP, GMP or XMP

Eluant	Immobilised dye	
	Procion Red HE-3B	Procion Blue H-B *
AMP	5 5 (65)	1 4 (78)
NAD^+	4 5 (55)	3 3 (74)
IMP	—	32 (101)
GMP	—	25 (74)
XMP	—	20 (68)

* Cibacron Blue F3G-A

TABLE II

CHROMATOGRAPHY OF *E COLI* ADENYLOSUCCINATE SYNTHETASE ON IMMOBILISED TRIAZINE DYES

Elution was effected with a linear gradient of IMP (0—20 mM, 20 ml total volume) in 50 mM Tris-HCl pH 7 5 containing 1.5 mM $MgCl_2$ and 1 mM dithiothreitol

Immobilised dye	[Dye] (μmol/g moist wt.)	Recovered adenylosuccinate synthetase activity (%)				[IMP] required to elute peak activity of enzyme (mM)	Capacity (units in IMP gradient)
		Buffer wash	IMP gradient	1 M KCl	Total recovery		
Yellow H-A	1 1	102	0	0	102	—	0
H-5G	2 7	0	32	0	32	1 6	1 6
MX-R	8 3	50	58	0	108	0.5	0 9
MX-8G	3 3	50	0	0	50	—	0
Brown H-2G	0 6	11	65	0	76	1 8	14 5
Orange MX-G	6 3	11	13	0	24	1 6	0 3
Scarlet MX-G	5.1	10	33	59	102	2 4	0 8
Rubine MX-B	1 9	9	47	47	103	2 0	3 1
Red H-3B	1 4	0	87	0	87	1.4	7 5
HE-3B	1 0	26	72	0	98	0 7	9 2
MX-5B	8 7	0	16	43	59	1 7	0 3
Blue MX-R	6 7	6	13	14	33	1 7	0.3
H-B *	1 0	11	26	55	92	0 9	3 3
Green HE-4BD	2 5	0	27	59	86	9 5	1 4

* Cibacron Blue F3G-A

F3G-A. The tight binding and high capacities observed for immobilised blue, green and red dyes has also been observed for *E coli* IMP dehydrogenase [14] and pig heart lactate dehydrogenase [25]. The overall recovery of enzyme activity typically fell within the range 24—108%, depending on the immobilised dye [14]. The adsorbents bearing Procion Red HE-3B, Red H-3B and Cibacron Blue F3G-A exhibited the highest capacity for *E coli* adenylosuccinate synthetase in terms of the recovered units of enzyme activity/μmol immobilised dye. Similar behaviour has been noted for *E coli* IMP dehydrogenase [14] and these dyes were therefore selected for further study.

Kinetic inhibition studies with adenylosuccinate synthetase

Kinetic inhibition studies were performed with *E coli* adenylosuccinate synthetase and Procion Red H-3B and Cibacron Blue F3G-A versus IMP, GTP and L-aspartate as variable substrates. For both Procion Red H-3B and Cibacron Blue F3G-A, *E coli* adenylosuccinate synthetase displayed strictly competitive inhibition with respect to GTP, mixed-type inhibition with respect to IMP and non-competitive inhibition with respect to L-aspartate. The two dyes, Cibacron Blue F3G-A and Procion Red H-3B thus appear to be able to discriminate between the binding sites of the three substrates, IMP, GTP and L-aspartate on *E coli* adenylosuccinate synthetase. The kinetic inhibition constants (K_i) are summarised in Table III and reveal that the enzyme has a considerably higher affinity for Cibacron Blue F3G-A than for Red H-3B, an observation also found for IMP dehydrogenase (Table III).

TABLE III

KINETIC CONSTANTS OF TRIAZINE DYES FOR *E COLI* IMP DEHYDROGENASE AND ADENYLOSUCCINATE SYNTHETASE

The type of inhibition is c, competitive, m, mixed, nc, non-competitive The concentrations of the dyes were determined by absorbance using the following molar extinction coefficients Procion Blue H-B (Cibacron Blue F3G-A), 13 300 l mol^{-1} cm^{-1}, Procion Red HE-3B, 30 100 l mol^{-1} cm^{-1}, Procion Red H-3B, 18 100 l mol^{-1} cm^{-1}

Enzyme	Substrate	K_m (μM)	Inhibitor K_i (μM) (Type of inhibition)		
			Blue H-B	Red HE-3B	Red H-3B
IMP dehydrogenase	IMP	9 4	5 7 (c)	— (m)	—
	NAD^+	390 2	24 4 (m)	114 3 (c)	—
Adenylosuccinate synthetase	IMP	16.7	0 5 (m)	—	6 4 (m)
	GTP	11 5	0 5 (c)	—	13 2 (c)
	L-Aspartate	1270	1 4 (nc)	—	54 0 (nc)

Biospecific elution of E coli adenylosuccinate synthetase

When the enzyme was chromatographed on agarose-immobilised Cibacron Blue F3G-A, any single substrate or any combination of two substrates resulted in poor enzyme activity recoveries (26—34%, Table IV). This may reflect the low inhibition constants of Cibacron Blue F3G-A versus IMP, GTP and L-aspartate (0.5, 0.5 and 1.4 μM, respectively, Table III) compared to the Michaelis constants for the same substrates, 16.7, 11.5 and 1270 μM respectively (Table III). In contrast, nearly quantitative elution of adenylosuccinate synthetase activity (86%) from an immobilised Cibacron Blue F3G-A (Procion Blue H-B) column could be effected in the presence of all three substrates (Table IV). These observations concur with those reported for lactate dehydrogenase and 3-phosphoglycerate kinase where elution from blue dextran-agarose columns was facilitated with 1 mM NAD^+ plus 10 mM pyruvate or ATP plus 3-phosphoglycerate respectively but not by the substrates individually [4,26].

TABLE IV

SPECIFIC ELUTION OF *E COLI* ADENYLOSUCCINATE SYNTHETASE FROM IMMOBILISED TRIAZINE DYES

A sample (200 μl, 6 5 units adenylosuccinate synthetase) was applied to a column (0.45 × 3 2 cm, 0 5 g moist weight gel, 0 8 μmol Procion Blue H-B/g moist weight gel), washed with 10—12 ml 50 mM Tris-HCl, pH 7 5, containing 1 5 mM $MgCl_2$ and 1 mM dithiothreitol. Enzyme activity was eluted by applying a pulse (5 ml) of 5 mM of each of the eluants below Chromatography was performed at 23—25°C

Eluant (5 mM)	Eluted enzyme activity (%)
IMP	26
GTP	32
L-Aspartate	0
IMP + L-Aspartate	28
GTP + L-Aspartate	32
IMP + GTP	34
IMP + GTP + L-Aspartate	86

TABLE V

LARGE SCALE PURIFICATION OF ADENYLOSUCCINATE SYNTHETASE FROM *E COLI purA* MUTANT MW1068

Purification stage	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (-fold)
1 Cell free extract	50	2380	5290	2 2	100	1 0
2 Chromatography on DEAE-cellulose, 0—50% satd $(NH_4)_2SO_4$	7 1	284	4786	16 8	90	7 6
3 Procion Red H-3B agarose	2 8	16 8	2128	127 0	40	57 7
4 Procion Blue H-B agarose	1 8	3 6	1829	508.0	35	231 0

Large scale purification of E coli adenylosuccinate synthetase

Affinity chromatography on immobilised triazine dyes may be exploited to purify *E coli* adenylosuccinate synthetase with a higher specific activity than that previously reported [27,28]. The cell-free extract arising from a 40 l culture of *E coli* MW1068 was subjected to a preliminary chromatography on DEAE-cellulose with subsequent elution with a linear gradient of NaCl (Table V). The fractions containing adenylosuccinate synthetase activity eluted at approximately 0.15 M NaCl and were pooled and precipitated immediately with 50% saturated $(NH_4)_2SO_4$. After dialysis a 7.6-fold enhancement in specific activity was observed with a 90% overall yield whence the crude enzyme preparation could be applied to an immobilised Procion Red H-3B column and eluted with a linear gradient of salt. The pooled concentrated and dialysed fractions were applied to an immobilised Cibacron Blue F3G-A (Procion Blue H-B) column and enzyme activity eluted with nearly 90% recovery with a specific eluant mixture comprising IMP, GTP and L-aspartate. Specific elution of adenylosuccinate synthetase by IMP from columns of Procion Red H-3B was not used on a preparative scale, despite being efficient on a small scale (Table II), since recoveries of enzyme activity were significantly reduced on scale-up of the chromatographic procedures. The overall purification scheme summarised in Table V affords 3.6 mg adenylosuccinate synthetase of specific activity 508 units/mg (determined at 25°C) and compares favourably with the purification schemes of Lieberman [27] and Eyzaguirre and Atkinson [28], resulting in specific activities of 66 (37°C) and 77 units/mg (30°C), respectively. The overall yield of enzyme activity reported here (35%) also compares favourably with any procedure published so far for the *E coli* enzyme [27,28].

Conclusions

Over the past few years substantial evidence has accrued to demonstrate unequivocally that triazine dyes interact with nucleotide-binding sites on enzymes [1,3—9,11,29]. The present data suggests that, in addition, triazine dyes may be able to discriminate between nucleotide-binding sites on the same enzyme. Thus, studies by kinetic inhibition, enzyme inactivation and specific elution techniques indicate that triazine dyes such as Cibacron Blue F3G-A (Procion Blue H-B), Procion Red HE-3B and Procion Red H-3B are able to dif-

ferentiate between the nucleotide-binding sites of *E coli* IMP dehydrogenase and adenylosuccinate synthetase. In contrast, earlier studies have shown that Cibacron Blue F3G-A displayed non-competitive inhibition relative to the ATP, UTP and template poly[d(A-T)] binding sites of *Azotobacter vinelandii* RNA polymerase [30] and towards the NAD^+- and ATP-binding sites of pigeon liver NAD^+ kinase [31]. It is difficult to rationalise these observations without speculating that Cibacron Blue F3G-A may be a better analogue of monophosphates such as IMP than di- or triphosphate nucleotides such as NAD^+ or ATP. In this context, it has been suggested that Procion Red HE-3B displays some preference for $NADP^+$-dependent dehydrogenases whilst Cibacron Blue F3G-A may preferentially bind NAD^+-dependent enzymes [5,15,29] although this may be by no means a general rule [32]. The most likely explanation for these data is that these dyes are not highly specific nucleotide or coenzyme analogues and that they do not assume a single unique conformation on different enzymes or on different nucleotide-binding sites on the same enzyme [9]. This conclusion is substantiated by the fact that a number of dyes of grossly different chemical structures can apparently bind to the coenzyme-binding site of lactate dehydrogenase [7,25] with a wide range of affinities. Not surprisingly, therefore, triazine dyes might be able to, at least partially, differentiate between related nucleotide-binding sites on the same enzyme. Information of this kind is useful for the design of specific elution regimes for large scale affinity chromatography on immobilised Procion Dyes [29].

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