Triazine dyes, a new class of affinity labels for nucleotide-dependent enzymes

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A number of reactive dichlorotriazine dyes specifically and irreversibly inactivate pig heart lactate dehydrogenase, yeast glucose 6-phosphate dehydrogenase and yeast hexokinase at sites competitive with NAD⁺, NADP⁺ and ATP respectively. Monochlorotriazine dyes, including Cibacron Blue F3G-A, do not inactivate lactate dehydrogenase but display high affinity and thus inhibit the inactivation by dichlorotriazine dyes. These data are interpreted in terms of the ability of nucleotide-binding enzymes to bind polysulphonated aromatic chromophores.

Immobilized Cibacron Blue F3G-A and other triazine-based dyes have received increasing attention over the last decade or so as 'group-specific' adsorbents for the purification of a wide range of enzymes by affinity chromatography (Böhme *et al.*, 1972; Travis & Pannell, 1973; Angal & Dean, 1978; Stellwagen, 1977; Dean & Watson, 1979; Lowe, 1979). Not surprisingly, therefore, a number of studies have been initiated to establish the basis for these selective interactions (Ashton & Polya, 1978; Beissner & Rudolph, 1978; Edwards & Woody, 1979). Thus, it has been suggested that the polysulphonated aromatic chromophores of the triazine dyes mimic the naturally occurring biological heterocycles such as nucleotide mono-, di- and triphosphates, NAD⁺, NADP⁺, flavins, acetyl-CoA and folic acid (Jacobsberg *et al.*, 1975; Thompson *et al.*, 1975; Baird *et al.*, 1976; Edwards & Woody, 1979). However, more recent spectroscopic, kinetic and X-ray diffraction studies suggest that the triazine dyes are not highly specific coenzyme or nucleotide analogues since only part of the chromophore accurately mimics coenzyme binding to the complementary enzymes (Ashton & Polya, 1978; Biellmann *et al.*, 1979; Edwards & Woody, 1979).

Since there are a number of reactive dyes that contain mono- or dichlorotriazinyl functional groups and which, at least in part, mimic coenzyme binding, it is not unreasonable to suppose that the free dyes might prove to be effective irreversible affinity labels for nucleotide-dependent enzymes. The present report demonstrates that a number of triazine dyes irreversibly inactivate typical nucleotide-dependent

enzymes with similar rates of maximal inactivation but with markedly different affinities.

Experimental

Materials

The Procion dyes used in this study were a generous and much appreciated gift from Dr. C. V. Stead, ICI Organics Division, Blackley, Manchester, U.K. Pig heart lactate dehydrogenase (L-lactate-NAD⁺ oxidoreductase, EC 1.1.1.27; 10 mg/ml, 220 units/mg at 25°C), NADH and ATP were purchased from Boehringer whereas yeast hexokinase (ATP-D-hexose 6-phosphotransferase, EC 2.7.1.1; 310 units/mg), yeast glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate-NADP⁺ oxidoreductase, EC 1.1.1.49; 315 units/mg), D-glucose 6-phosphate, AMP, NAD⁺ and NADP⁺ were from Sigma. Sodium pyruvate and all other chemicals were obtained from BDH.

Methods

All enzymes were assayed at 25°C in a Pye-Unicam SP.1800 spectrophotometer as described by Bergmeyer (1974). One unit of enzyme activity is defined as the amount that catalyses the conversion of 1 μ mol of substrate to product/min at 25°C.

Enzyme inactivation by free triazine dyes was performed in 0.1 M-Tris/HCl buffer, pH 8.5 at 35°C. The reaction vial contained in a total volume of 1 ml: enzyme (1 unit, 25°C), triazine dye (0–200 nmol) and Tris/HCl buffer, pH 8.5 (100 μ mol). The rate of dye inactivation was followed by periodically removing samples (20 μ l) and assaying for enzyme activity. Initial rates of inactivation were deduced from plots of log₁₀(% of activity remaining) versus

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time (min) for several dye concentrations and the slopes and intercepts of secondary double reciprocal plots were calculated by unweighted linear regression analysis on a Hewlett-Packard model 9810 programmable calculator.

The reaction mixture protocol for the determination of the dissociation constants of the monochlorotriazinyl (H-type) dyes at 35°C in a total volume of 1 ml included: pig heart lactate dehydrogenase (1 unit, 25°C), Procion Blue MX-R (30 nmol), monochlorotriazinyl dye (30–60 nmol), Tris/HCl buffer, pH 8.5 (100 μmol). Dye concentrations were determined spectrophotometrically (Ashton & Polya, 1978).

Results

The reaction between an active-site-directed reactive dye (D) and an enzyme (E) may be formulated by:

where $E \cdot D$ is the enzyme-dye Michaelis complex, ED is the irreversibly inhibited enzyme and k_3 is the

Fig. 1. Time course for the inhibition of pig heart lactate dehydrogenase by several representative triazine dyes at pH 8.5 and 35°C

Procion Red H-3B (○); Red HE-3B (▲); Yellow H-A (●); Blue H-B (△); Yellow MX-8G (■); Scarlet MX-G (◐); Yellow MX-R (□); Blue MX-R (◑). The concentration of each dye was 200 μM.

rate-limiting step. A steady-state treatment of the process yields the equation:

$$\frac{1}{k_{\text{obs.}}} = \frac{1}{k_3} + \frac{K_D}{k_3} \cdot \frac{1}{[D]} \quad (2)$$

where $k_{\text{obs.}}$ is the observed rate of enzyme inactivation for a given concentration of dye, D , k_3 is the maximal rate of inactivation (min^{-1}) and K_D is the dissociation constant (k_2/k_1) of the enzyme-dye complex (Kitz & Wilson, 1962; Witt & Roskoski, 1980).

The inactivation of lactate dehydrogenase by several representative triazine dyes is shown in Fig. 1. Of the dyes tested (Table 1) significant inactivation rates were only obtained with dichlorotriazinyl dyes of MX designation. In contrast, monochlorotriazinyl dyes of designation H, HE or P do not significantly inactivate lactate dehydrogenase even with dye concentrations as high as 200 μM. Fig. 2 demonstrates that lactate dehydrogenase is rapidly inactivated by 30 μM-Procion Blue

Fig. 2. Effect of competing nucleotides on the time course for the inactivation of pig heart lactate dehydrogenase by Procion Blue MX-R (30 μM) at pH 8.5 and 35°C
No addition (●); NADH, 1 mM (○); NAD⁺, 1 mM (▲); AMP, 1 mM (△).

MX-R, but that the rate of inactivation is decelerated in the presence of 1mM-AMP and NAD⁺ and totally extinguished by 1mM-NADH. Competitive inhibitors characteristically decrease the rate of enzyme inactivation by active-site-directed reagents according to the equation (Kitz & Wilson, 1962; Witt & Roskoski, 1980):

$$\frac{1}{k_{obs.}} = \frac{1}{k_3} + \frac{K_D}{k_3} \cdot \frac{1}{[D]} \left(1 + \frac{[I]}{K_I} \right) \quad (3)$$

where I is the competitive inhibitor and K_I its dissociation constant. The inactivation of hexokinase by 100μM-Procion Blue MX-R and glucose 6-phosphate dehydrogenase by 10μM-Procion Blue MX-R was likewise completely inhibited by 20mM-ATP and 20mM-NADP⁺ respectively. The inactivation of lactate dehydrogenase was irreversible since no activity could be recovered by subsequent incubations with high concentrations (10mM) of NADH. Furthermore, the inactivated enzyme

remains coloured even after passage through a Sephadex G-25 column or after exhaustive dialysis. The inactivation of lactate dehydrogenase, hexokinase and glucose 6-phosphate dehydrogenase by dichlorotriazinyl dyes at pH8.5 and 35°C follows approximately pseudo-first-order kinetics at low dye concentrations. The range of dye concentrations where approximate linearity in semi-logarithmic plots is observed varies from enzyme to enzyme for the same dye and from dye to dye for the same enzyme but generally lies within the range 0–100μM. However, even at these dye concentrations some deviation from linearity occurs beyond about 75% enzyme inactivation and hence in most cases k_{obs.} was calculated from the initial linear proportion of the curves. These curving semi-logarithmic plots are presumably the result of inactivation of the reactive dye in the incubation medium, of dye aggregation and of reaction with the enzyme at points other than the active site region. Nevertheless, the double reciprocal plot (Fig. 3) of 1/k_{obs.} versus 1/[D] for several representative dyes yields a straight line with a positive ordinate intercept and is indicative of saturation kinetics for the inactivation process. The maximum rate of inactivation (k₃)

Fig. 3. Effect of dye concentration on the observed rate of inactivation (k_{obs.}) of lactate dehydrogenase by several dichlorotriazine dyes expressed as a double reciprocal plot
Procion Scarlet MX-G (●); Orange MX-G (■); Yellow MX-R (○); Rubine MX-B (△); Blue MX-R (▲).

Table 1. Maximal rates of inactivation (k₃) and dissociation constants (K_D) of Procion dyes for pig heart lactate dehydrogenase

The inactivation of lactate dehydrogenase by triazine dyes was performed in 0.1M-Tris/HCl buffer, pH8.5 at 35°C, and the above parameters were deduced as described in the Experimental section.

Procion dye	k ₃ (min ⁻¹)	K _D (μM)
Blue HE-RD	—	0.8
Green HE-4BD	—	1.8
Green H-4G	—	2.5
Blue H-B*	—	5.6
Brown H-2G	—	6.1
Blue MX-R†	0.12	7.2
Yellow H-5G	—	7.3
Red H-3B	—	10.1
Red HE-3B	—	10.1
Turquoise H-A	—	13.4
Violet H-3R	—	15.6
Red P-3BN	—	20.0
Rubine MX-B	0.22	31.9
Red MX-5B	0.09	32.2
Yellow H-A	—	36.0
Yellow MX-R	0.16	72.8
Scarlet MX-G	0.09	88.9
Orange MX-G	0.12	93.4
Yellow MX-8G	0.13	122.9

* Cibacron Blue F3G-A.
† For hexokinase and glucose 6-phosphate dehydrogenase, k₃ was 0.08 and 0.30min⁻¹, and K_D was 7.5 and 4.8 μM respectively under the same conditions.

Fig. 4. Effect of competing monochlorotriazinyl dyes on the time course for the inactivation of pig heart lactate dehydrogenase by Procione Blue MX-R at pH 8.5 and 35°C

All incubations contained Procione Blue MX-R (30 μ M) supplemented with the following dyes: Procione Blue HE-RD, 30 μ M (Δ); Green H-4G, 30 μ M ($\blacktriangle$); Blue H-B, 60 μ M ($\square$); Red H-3B, 60 μ M ($\blacksquare$); Turquoise H-A, 30 μ M ($\bullet$); none ($\circ$).

determined from the ordinate intercept for the dichlorotriazinyl (MX) dyes listed in Table 1 is remarkably consistent at 0.09–0.22 min⁻¹. The dissociation constants (K_D) for these dichlorotriazinyl dyes determined from the slopes of the double reciprocal plots are also listed in Table 1. The dissociation constants of the monochlorotriazinyl H-, HE- and P-type dyes were calculated from data inserted into eqn. (3) and obtained by competition between Procione Blue MX-R and the monochlorotriazinyl dyes (Fig. 4). The rate of inactivation of lactate dehydrogenase by monochlorotriazinyl dyes was negligible compared with the rate of inactivation by dichlorotriazinyl dyes under the conditions cited (Fig. 1). The dissociation constants for triazine dyes (Table 1) encompass a 154-fold range from 0.8–122.9 μ M, are lowest for blue-green dyes and highest for yellow-orange dyes and, with one or two exceptions, are lower for H-type dyes than MX-type dyes.

Discussion

Reactive dichlorotriazine dyes of the Procione MX-range inactivate lactate dehydrogenase, hexo-

kinase and glucose 6-phosphate dehydrogenase in a time-dependent fashion. The hyperbolic dependence of inhibition of dye concentration, the protection against inhibition afforded by specific nucleotides and the lack of reactivation after inhibition are characteristic of active-site-directed irreversible inhibitors (Kitz & Wilson, 1962; Witt & Roskoski, 1980). Furthermore, despite considerable structural disparity between the MX-dyes investigated (Ashton & Polya, 1978), the rates of maximum inactivation (k_3) of lactate dehydrogenase differed by only 2.4-fold (0.09–0.22 min⁻¹) overall, whereas the dissociation constants (K_D) for the same dyes varied 17.1-fold (7.2–122.9 μ M). This observation suggests that the reactive dichlorotriazine functional group of each dye is positioned close to a nucleophile in the coenzyme-binding site. A potential candidate for this nucleophile might be the ϵ -amino group of lysine-58, which lies close to the pyrophosphate bridge and the O3' of the adenosine ribose moiety (Holbrook *et al.*, 1975) and close to where X-ray diffraction studies place the triazine ring of Cibacron Blue F3G-A in liver alcohol dehydrogenase (Biellmann *et al.*, 1979). Furthermore, the similarity in the behaviour of the three enzymes tested towards inhibition by Procione Blue MX-R (Table 1) suggests that this region of the coenzyme-binding site might be conserved.

Monochlorotriazine dyes of the Procione H-, HE- or P-ranges are markedly less reactive than the corresponding dichlorotriazines and thus fail to inactivate lactate dehydrogenase under the conditions cited. Nevertheless, Cibacron Blue F3G-A does inhibit cyclic AMP-dependent protein kinase by covalent modification (Witt & Roskoski, 1980). Monochlorotriazinyl dyes do, however, display marked affinity for lactate dehydrogenase with dissociation constants considerably lower than the coenzyme, NAD⁺, itself. It has been suggested that the affinity of Cibacron Blue F3G-A for the enzyme may be enhanced because it resembles the NAD-pyruvate adduct and may thus be a transition state analogue (Thompson & Stellwagen, 1976). This hypothesis is unlikely in view of the fact that several structurally unrelated dyes (Table 1) display higher affinity for lactate dehydrogenase than does Cibacron Blue F3G-A. It seems more likely that the topography of the coenzyme binding site in this enzyme allows some latitude in binding polysulphonated aromatic chromophores.

References

- Angal, S. & Dean, P. D. G. (1978) *FEBS Lett.* **96**, 346–348
- Ashton, A. R. & Polya, G. M. (1978) *Biochem. J.* **175**, 501–506
- Baird, J. K., Sherwood, R. F., Carr, R. J. G. & Atkinson, A. (1976) *FEBS Lett.* **70**, 61–66

- Beissner, R. S. & Rudolph, F. B. (1978) *J. Chromatogr.* **161**, 127–135
- Bergmeyer, H. U. (1974) *Methods of Enzymatic Analysis*, vol. 1, Verlag-Chemie/Academic Press, New York
- Biellmann, J.-F., Samama, J.-P., Brändén, C. I. & Eklund, H. (1979) *Eur. J. Biochem.* **102**, 107–110
- Böhme, H., Kopperschläger, G., Schulz, J. & Hofmann, E. (1972) *J. Chromatogr.* **69**, 209–214
- Dean, P. D. G. & Watson, D. H. (1979) *J. Chromatogr.* **165**, 301–319
- Edwards, R. A. & Woody, R. W. (1979) *Biochemistry* **18**, 5197–5204
- Holbrook, J. J., Liljas, A., Steindel, S. J. & Rossmann, M. G. (1975) *Enzymes 3rd Ed.* **11A**, 191–292
- Jacobsberg, L. B., Kantrowitz, E. R. & Lipscomb, W. N. (1975) *J. Biol. Chem.* **250**, 9238–9249
- Kitz, R. & Wilson, I. B. (1962) *J. Biol. Chem.* **237**, 3245–3249
- Lowe, C. R. (1979) in *An Introduction to Affinity Chromatography: Laboratory Techniques in Biochemistry and Molecular Biology* (Work, T. S. & Work, E., eds.), pp. 453–456, North-Holland, Amsterdam
- Stellwagen, E. (1977) *Acc. Chem. Res.* **10**, 92–98
- Thompson, S. T. & Stellwagen, E. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 361–365
- Thompson, S. T., Cass, K. H. & Stellwagen, E. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 669–672
- Travis, J. & Pannell, R. (1973) *Clin. Chim. Acta* **49**, 49–52
- Witt, J. J. & Roskoski, R. (1980) *Biochemistry* **19**, 143–148