Fig. 2) yet for the evaluation of our binding data a model considering a single specific and a nonspecific class of binding sites on the human α_1-AGP for propranolol was utilized. This model can be validated only indirectly, because no information on the binding sites of human α_1-AGP for propranolol at molecular level is available as yet. According to Klotz [12], the semilogarithmic plot $B = f(F)$, illustrating the binding of a ligand to specific sites, is an S-shaped curve with the inflection point appearing at half-maximum binding. Considering our results shown in Fig. 2 in terms of this mode of presentation, the total number of sites originating exclusively in specific drug–protein interactions is to be rejected.

The present paper demonstrates that HPLC is an efficient method for studying the binding of propranolol to human α_1-acid glycoprotein. Considering a single specific and a nonspecific class of binding sites on human α_1-AGP for propranolol the estimated mean number of specific binding sites was $n = 0.81$, with an affinity of $k = 5.4 \times 10^5\ M^{-1}$, and for the nonspecific binding their product $n'k'$ was $7.4 \times 10^3\ M^{-1}$.

Acknowledgements—The author L.Š. is very grateful to the University of Paris—Val de Marne, and to the Scientific Exchange Agreement—Foundation Hamilton, for supporting his stage at the Laboratory of Pharmacology, Faculty of Medicine, and at the Laboratory of Physical Chemistry of Biopolymers, U. E. R. de Sciences, Créteil.

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REFERENCES

1. L. S. Goodman and A. Gilman (Eds.), *The Pharmacological Basis of Therapeutics*, 5th Edn, p. 551. Macmillan, New York (1975).
2. W. E. Müller and A. E. Stillbauer, *Naunyn-Schmiedeberg's Arch. Pharmac.* **322**, 170 (1983).
3. A. K. L. Wong and J. C. Hsia, *Can. J. Biochem. Cell Biol.* **61**, 1114 (1983).
4. F. M. Belpaire, R. A. Braeckman and M. G. Bogaert, *Biochem. Pharmac.* **33**, 2065 (1984).
5. B. Sebille, N. Thuaud and J.-P. Tillement, *J. Chromatogr.* **167**, 159 (1978).
6. B. Sebille, N. Thuaud and J.-P. Tillement, *J. Chromatogr.* **180**, 103 (1979).
7. B. Sebille, N. Thuaud and J.-P. Tillement, *J. Chromatogr.* **204**, 285 (1981).
8. S. F. Sun, S. W. Kuo and R. A. Nash, *J. Chromatogr.* **288**, 377 (1984).
9. C. M. Metzler, G. K. Elfring and A. J. McEwen, *Biometrics* **30**, 562 (1974).
10. J. P. Hummel and W. J. Dreyer, *Biochim. biophys. Acta* **63**, 530 (1962).
11. W. E. Müller, A. E. Stillbauer and S. El-Gamal, *J. Pharm. Pharmac.* **35**, 684 (1983).
12. I. M. Klotz, *Science* **217**, 1247 (1982).

Biochemical Pharmacology, Vol. 34, No. 24, pp. 4334–4337, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

Characterisation of a new transposon-mediated trimethoprim-resistant dihydrofolate reductase

(*Received* 30 *April* 1985; *accepted* 6 *August* 1985)

Plasmid-mediated resistance to trimethoprim (Tp) is due to the plasmid directed synthesis of additional Tp-insensitive dihydrofolate reductases (DHFR) (5,6,7,8-tetrahydrofolate: NADP+ oxidoreductase (EC 1.5.1.3)) [1, 2]. The resistant enzymes are much larger than the sensitive chromosomal DHFR with molecular weights (MW) of about 35,000 as compared to the *Escherichia coli* chromosomal enzyme which has a MW of only 21,000 [3, 4]. The plasmid encoded enzymes have since been classified into two major groups — type I and type II [3]. Although both enzyme types have a molecular weight of about 35,000 the subunit composition of the enzymes differs — type I enzymes consisting of two identical subunits molecular weight 18,000 and type II enzymes comprising four subunits molecular weight 9000 [5]. The enzymes also differ in several other properties. A third plasmid-mediated Tp-resistant DHFR (type III) has recently been reported by Fling *et al* [6]. This enzyme has monomeric structure, low molecular weight (16,900) and mediates only a moderate level of Tp resistance [7].

The type I DHFR is often encoded by a transposon (Tn7) which also encodes resistance to streptomycin [8]. The new 2 megadalton resistance transposon, Tn4132, conferring high level resistance to Tp alone, was extracted from a clinical plasmid isolated from a urinary pathogen in Edinburgh [9]. In this paper, the DHFR encoded by this transposon has been characterised and compared with the type I enzyme encoded by a Tn7-like transposon, Tn4130.

Materials and methods

Materials. The auxotrophic organism *Escherichia coli* K-12 strain J62 [10] harbouring plasmid RP4 [11] containing either Tn4130 or Tn4132 were used from our previous study [9]. NADPH and dihydrofolate were purchased from the Sigma Chemical Company (London) and Sephadex G-75 superfine from Pharmacia Fine Chemicals AB, Sweden. Trimethorpim lactate was kindly donated by the Wellcome Research Laboratories (Beckenham, U.K.).

Enzyme assay. Dihydrofolate reductase activity was assayed by the method of Osborn and Huennekens [12] as described previously [1]. Protein concentrations were estimated by the method of Waddell [13].

Enzyme preparation. The enzyme was prepared from ten litre batches of exponential phase bacteria by the method of Amyes and Smith [1]. Removal of NADPH oxidase activity and subsequent precipitation of DHFR activity was

achieved by dialysis for 4 hr against a 50% and then an 80% saturated solution of ammonium sulphate. Chromosomal and transposon determined enzymes were then separated by Sephadex exclusion chromatography by the method of Pattishall *et al.* [3].

Dihydrofolate reductase molecular weight determinations. Molecular weights were determined by Sephadex exclusion chromatography as described previously [1] with ovalbumin, chymotrypsinogen and cytochrome-*c* as standard markers.

Heat sensitivity. The enzyme preparation was maintained at 45° in buffer A (50 mM sodium phosphate buffer pH 7.4 containing 10 mM 2-mercaptoethanol and 1 mM EDTA) in a prewarmed container. After the requisite time, the sample was cooled in ice and the enzyme activity was assayed in the usual manner.

Polyacrylamide gel electrophoresis. The method used was that of Davis [14] as modified by Amyes and Smith [15]. Following electrophoresis the gels were sliced into 3 mm segments and each slice placed in 0.5 ml buffer A. The enzymes were allowed to elute overnight and the eluate was then assayed for DHFR activity.

Results

Purification and molecular weight determination of dihydrofolate reductase. The purification of DHFR from strains harbouring Tn4132 and Tn4130 (in parentheses) is shown in Table 1. Following gel filtration the peak fraction containing Tp resistant DHFR activity (Fig. 1) exhibited a 14-fold purification in the case of the Tn4132 mediated enzyme and a six fold purification in the case of the Tn4130 encoded DHFR (Table 1). Gel filtration in the presence of standard proteins revealed that the enzyme mediated by Tn4130 was 32,000 daltons (similar to the type I Tn7 encoded DHFR) whereas the enzyme encoded by Tn4132 was only 24,500 daltons in size (Table 2). This is much smaller than any previously described transposon mediated DHFR.

pH profiles, heat sensitivity and inhibition by antifolate compounds. The pH profiles of the partially purified enzymes were similar with a peak of activity at pH 6 and 95% activity over a range of one pH unit. Both enzymes were found to be heat labile and lost 50% activity within two minutes when maintained at 45° (TD_{50}) (Table 2). The activities of the two enzymes were re-assayed at pH 6 in the presence of increasing concentrations of Tp and methotrexate in order to determine the concentration of each antifolate required to give 50% inhibition (ID_{50}). The inhibitor profiles of both enzymes were very similar and the results (Table 2) show that both DHFRs were 50% inhibited by similar Tp and methotrexate concentrations.

Michaelis–Menten kinetics. The activities of the partially purified enzymes were investigated under conditions of partial saturation with dihydrofolate (DHF) and the results analysed by the method of Lineweaver and Burk. The results (Fig. 2) show that the K_m for DHF with the Tn4130 DHFR was 21 μM and with the Tn4132 DHFR was 11 μM and thus no significant difference could be demonstrated in the enzymes affinity for dihydrofolate.

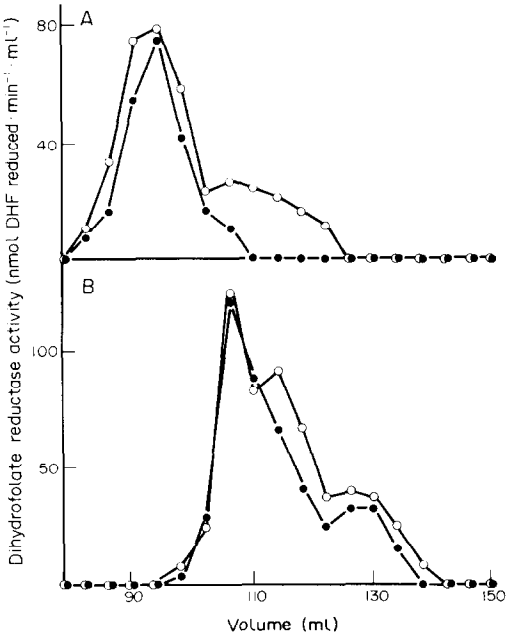

Fig. 1. Sephadex G-75 superfine gel filtration. The dihydrofolate reductase activities obtained after Sephadex exclusion chromatography are shown assayed in the presence and absence of trimethoprim. (A) Preparation obtained from *E. coli* J62(RP4::Tn4130) after precipitation with 50–80% saturation ammonium sulphate. (B) Preparation obtained from *E. coli* J62 (RP4::Tn4132) after precipitation with 50–80% saturation ammonium sulphate. (○) No trimethoprim; (●) 4 μM trimethoprim.

In addition, when the assays were repeated in the presence of Tp it was found that the maximum velocity remained the same for both enzymes (Fig. 2) indicating that the drug causes competitive inhibition with both DHFRs. The inhibitor constants (K_i) for the drug (as described by Dixon and Webb [16]) were 4.8 and 41.0 μM for the Tn4130 and Tn4132 DHFRs, respectively. Thus both enzymes have a very low affinity for Tp similar to the type I DHFRs [3, 4].

Polyacrylamide gel electrophoresis. The enzymic properties of the Tn4132 DHFR indicate that it is biochemically very similar to the type I, Tn4130 encoded enzyme.

Table 1. Purification of the dihydrofolate reductase activity from E. coli J62(RP4::Tn4132)

Stage	Volume (ml)	Protein conc. (mg/ml)	DHFR activity (U/ml)	Specific activity (U/mg protein)	Purification -fold	Recovery (%)
Bulk	16.5 (16.5)	65.3 (71.8)	297.7 (496.2)	4.5 (6.9)	1.0 (1.0)	100 (100)
50% $(NH_4)_2SO_4$ supernatant	7.5 (6.0)	40.2 (31.6)	206.8 (330.8)	5.1 (10.5)	1.1 (1.5)	31.6 (24.2)
Peak Sephadex fraction	2.0 (2.0)	1.9 (1.7)	124.1 (78.6)	62.6 (45.7)	13.7 (6.6)	5.1 (1.9)

Note: The values in parentheses represent results of the purification of DHFR activity from *E. coli* J62(RP4::Tn4130). The units of dihydrofolate reductase activity are nmoles dihydrofolate reduced/min in 40 mM sodium phosphate buffer pH 6.0 at 30°.

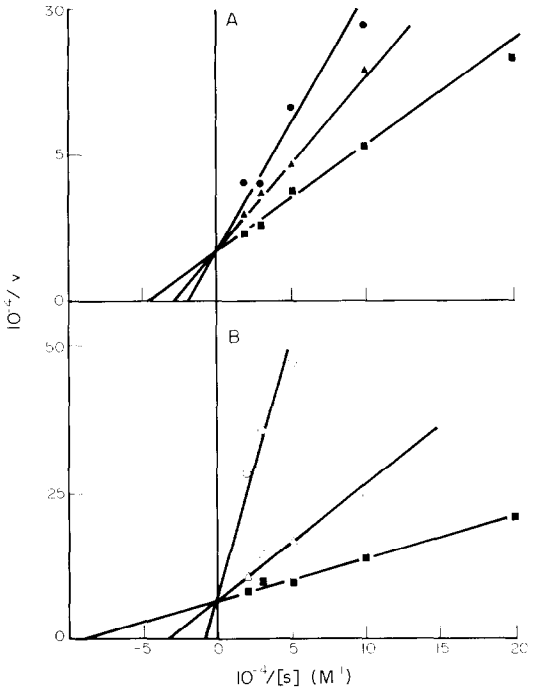

Fig. 2. Lineweaver–Burk plots. The reciprocal of the dihydrofolate reductase concentration (1/[s]) is plotted against the reciprocal of the rate of dihydrofolate reductase activity (1/v) in the presence and absence of trimethoprim. (A) A partially purified preparation of dihydrofolate reductase from *E. coli* J62(RP4::Tn4130). (B) A partially purified preparation of dihydrofolate reductase from *E. coli* J62(RP4::Tn4132). (■) No trimethoprim; (●) 40 μM; (▲) 4 μM; (○) 400 μM; (△) 80 μM trimethoprim.

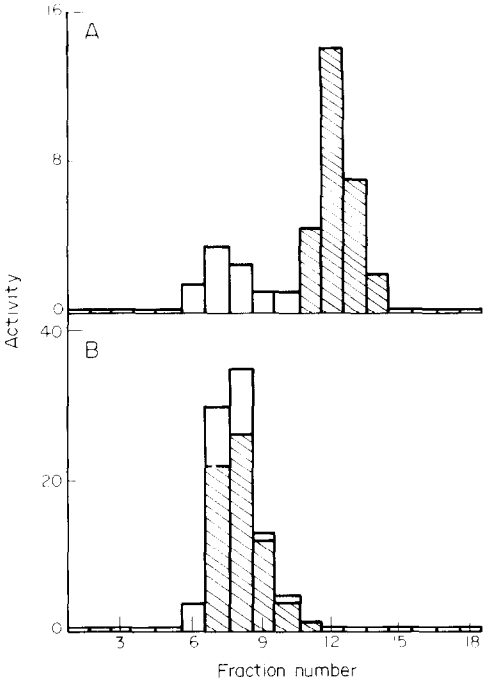

Fig. 3. Polyacrylamide gel electrophoresis. The units of dihydrofolate reductase activity eluted from slices after polyacrylamide gel electrophoresis are shown. Fraction 1 contains the bromophenol blue marker and the start of the running gel is fraction 18. (A) Ammonium sulphate precipitate obtained from an *E. coli* J62(RP4::Tn4130) crude extract after saturation with 50–80%. (B) Ammonium sulphate precipitate obtained from an *E. coli* J62(RP4::Tn4132) crude extract after saturation with 50–80%. The shaded areas show the level of dihydrofolate reductase activity in the presence of 4 μM trimethoprim.

Table 2. Properties of the dihydrofolate reductases coded by Tn4130 and Tn4132

Source of enzyme	Size (daltons)	TD_{50} at 45° (min)	ID_{50} of Tp (μM)	ID_{50} of Mtx* (μM)
Tn4130	32,000	1.4	63	6.3
Tn4132	24,500	1.2	32	2.8

* Mtx. = methotrexate.

However, it is distinct from previously described type I enzymes by its unusually low molecular weight. In order to confirm that the Tn4132 enzyme does indeed differ in molecular size from the Tn4130 DHFR, 80% ammonium sulphate precipitates of the enzymes produced by strains J62(RP4::Tn4130) and J62(RP4::Tn4132) were subjected to polyacrylamide gel electrophoresis as described. The results (Fig. 3) show that the sample from the Tn4130 containing strain exhibits two separate peaks of DHFR activity; a small peak of Tp sensitive chromosome determined DHFR activity in fraction 7 and a second much larger peak of Tp resistant transposon mediated DHFR activity eluting from fraction 12 (Fig. 3A). The sample from strain J62(RP4::Tn4132), on the other hand, shows a single peak of DHFR activity eluting from fraction 8, 75% of which is resistant to 4×10^{-6} M Tp (Fig. 3B). This indicates that three quarters of the activity in this peak represents the Tn4132 encoded enzyme, the remaining quarter corresponding to the host chromosomal enzyme. The absence of a second peak of DHFR activity at fraction 12 supports the view that the two transposons do indeed encode DHFRs of different molecular size, the enzyme produced by Tn4132 being closer in size to the host chromosomal enzyme than the enzyme encoded by Tn4130.

Discussion

The Tp resistant DHFR encoded by Tn4132 is biochemically very similar to the Tn4130 encoded type I DHFR but differs markedly in its molecular weight. It is synthesised in large amounts, is heat labile and is 50% inhibited by levels of Tp and methotrexate similar to those required for type I DHFR inhibition. However, the DHFR characterised here has a molecular weight of only 24,500 compared with the 32,000 daltons of the Tn4130 type I enzyme. In view of the strong enzymic similarity between the Tn4132 mediated DHFR and type I enzymes, we suggest that these two enzymes must be closely related, the smaller enzyme possibly consisting of only part of the two subunits of the type I DHFRs. We propose that this 'new' enzyme be classified as an unusual type I DHFR and believe we may be witnessing the continued evolution of these enzymes.

Acknowledgements—We wish to thank the Medical Research Council for their generous support of this work. We also acknowledge the Davidson Research Scholarship awarded to H-K. Young.

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REFERENCES

1. S. G. B. Amyes and J. T. Smith, *Biochem. biophys. Res. Commun.* **58**, 412 (1974).
2. O. Sköld and A. Widh, *J. biol. Chem.* **249**, 4324 (1974).
3. K. H. Pattishall, J. Acar, J. J. Burchall, F. W. Goldstein and R. J. Harvey, *J. biol. Chem.* **252**, 2319 (1977).
4. S. G. B. Amyes and J. T. Smith, *J. gen. Microbiol.* **107**, 263 (1978).
5. M. E. Fling and L. P. Elwell, *J. Bacteriol.* **141**, 779 (1980).
6. M. E. Fling, L. Walton and L. P. Elwell, *Antimicrob. Agents Chemother.* **22**, 882 (1982).
7. S. S. Joyner, M. E. Fling, D. Stone and D. P. Baccanari, *J. biol. Chem.* **259**, 5851 (1984).
8. P. T. Barth, N. Datta, R. W. Hedges and N. J. Grinter, J. Bacteriol. **125**, 800 (1976).
9. H-K. Young and S. G. B. Amyes, *Proceedings of 13th International Congress of Chemotherapy*, Vienna (1983).
10. R. C. Clowes and D. Rowley, *J. gen. Microbiol.* **11**, 250 (1954).
11. N. Datta, R. W. Hedges, E. J. Shaw, R. B. Sykes and M. H. Richmond, *J. Bacteriol.* **108**, 1244 (1971).
12. M. J. Osborn and F. M. Huennekens, *J. biol. Chem.* **233**, 969 (1958).
13. W. J. Waddell, *J. Lab. clin. Med.* **48**, 311 (1956).
14. B. J. Davis, *Ann. N.Y. Acad. Sci.* **121**, 404 (1964).
15. S. G. B. Amyes and J. T. Smith, *Eur. J. Biochem.* **61**, 597 (1976).
16. M. Dixon and E. C. Webb, in *Enzymes* (1st Edn), p. 8. Longmans, Green and Co., London (1958).

Biochemical Pharmacology, Vol. 34, No. 24, pp. 4337–4340, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

Carbamylated acetylcholinesterase: Acceleration of decarbamylation by bispyridinium oximes

(*Received* 18 *December* 1984; *accepted* 16 *July* 1985)

Experimental animals can be protected against the lethal effects of organophosphorus anticholinesterase compounds by pretreatment with a carbamate (e.g. pyridostigmine) and therapy with atropine [1, 2]. The theory is that comparatively little phosphorylation of acetylcholinesterase (AChE*; EC 3.1.1.7) occurs, since the enzyme is already partially carbamylated by the carbamate. Rapid elimination of the organophosphate from the body is accompanied by spontaneous decarbamylation of the enzyme, so that sufficient active enzyme to maintain normal function is soon present [3]. In the meantime, atropine blocks the muscarinic receptor from the adverse effects of the elevated concentration of acetylcholine [3]. The level of protection afforded by carbamate prophylaxis can be enhanced by including an oxime in the therapy [1]. Presumably the oxime rapidly reactivates any phosphorylated AChE present to regenerate active AChE. However, it is conceivable that the oxime might also accelerate the spontaneous decarbamylation of carbamylated enzyme, with consequent beneficial effects on the outcome of the intoxication. It is therefore surprising that this question of accelerated decarbamylation has received little attention to date. Wilson *et al.* reported in 1960 [4] that 1 mM pyridine-2-aldoxime methyl iodide (2-PAM) had no effect on the rate of decarbamylation of dimethylcarbamyl-AChE from the electric eel. Since then there have been some suggestions that oximes can affect the rate of decarbamylation, but these are based on indirect evidence [5–7]. We have determined the effects of four oximes on the rate of decarbamylation of AChE from four sources, and report our results below. The four oximes are the monopyridinium oxime 2-PAM, the bispyridinium bis-oxime TMB-4 and two isomeric H-oximes, HS-6 and HI-6 [8]. The sources of AChE were electric eel, bovine erythrocytes (both commercial water-soluble powders), human erythrocytes (commercial membrane-bound enzyme) and rabbit brain (Triton–solubilised enzyme). Most experiments were done with dimethylcarbamyl-AChE, but some studies were made of methylcarbamyl-AChE. Both dimethylcarbamates (e.g. pyridostigmine) and methylcarbamates (e.g. physostigmine) are effective prophylactically *in vivo*. Some experiments were also done with two bispyridinium compounds lacking an oxime group. One of these, 1,1′-(1,3-propanediyl) bispyridinium dibromide, is an analogue of TMB-4 with hydrogen atoms instead of oxime groups. The other, SAD-128, is a compound which can protect experimental animals against some toxic effects of the organophosphate Soman, and which appears to interact allosterically with AChE [9].

Methods

Methods for studying the decarbamylation of AChE inhibited by neostigmine (a dimethylcarbamate) have been reported previously [10]. Similar methods were used for physostigmine. Decarbamylation was found to be relatively rapid in the case of eel AChE, and for this enzyme the reactivation was followed to near completion and the rate constant was determined by non-linear regression analysis of the data [11]. Most experiments were done in 2 mM phosphate–150 mM NaCl, pH 7.0, at 25°; in some cases NaCl was omitted. Corrections were made for non-enzymic hydrolysis of substrate.

Eel AChE was obtained from the Worthington Biochemical Corp. and stabilised in solution with gelatin (0.1%, w/v). Soluble bovine erythrocyte AChE (Type XII) and membrane-bound human erythrocyte AChE (Type XIII) were obtained from the Sigma Chemical Co. Whole rabbit brains were homogenised in 10 vol. 0.32 M sucrose at 4°, and the homogenate was centrifuged at 19,000 *g* for

* Abbreviations: AChE, acetylcholinesterase; 2-PAM, pyridine-2-aldoxime methyl iodide; TMB-4, pyridinium, 1,1′-(1,3-propanediyl)bis[4-(hydroxyimino)methyl]dibromide; HS-6, pyridinium, 1-[[[3-(aminocarbonyl)pyridinio]methoxy]methyl]-2-[(hydroxyimino)methyl]diiodide; HI-6, pyridinium, 1-[[[4-(aminocarbonyl)pyridinio]methoxy]methyl]-2-[(hydroxyimino)methyl]dichloride; SAD-128, pyridinium, 1,1′-[oxybis(methylene)]bis[4-(1,1-dimethylethyl)]dichloride; and DMB, 3,3-dimethyl-1-butanol.