Detection of inhibition of 5-aminoimidazole-4-carboxamide ribotide transformylase by thioinosinic acid and azathioprine by a new colorimetric assay

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The colorimetric assay for 5-aminoimidazole-4-carboxamide ribotide (AICAR) transformylase (phosphoribosylaminoimidazolecarboxamide formyltransferase; EC 2.1.2.3) has been extensively modified. The modified assay is based upon the short-term permanganate oxidation of the folate product, tetrahydrofolate (H_4 folate) to *p*-aminobenzoyl glutamate (pABG). The modified assay was used to detect the transformylase activity in crude extracts of peripheral-blood mononuclear cells (PBMCs). Azathioprine and its metabolite, thioinosinic acid (tIMP), are competitive inhibitors (with respect to AICAR) of the chicken liver transformylase and the transformylase from PBMCs of the MRL/lpr mouse, an animal model of systemic autoimmune disease. The K_i values of tIMP and azathioprine for the chicken liver enzyme are $39 \pm 4 \mu M$ and $120 \pm 10 \mu M$, whereas the K_i values for the enzyme from PBMCs of the MRL/lpr mouse are $110 \pm 20 \mu M$ and $90 \pm 14 \mu M$ respectively. The anti-inflammatory drugs ibuprofen and naproxen are also inhibitors of the transformylase.

INTRODUCTION

The colorimetric assay for 5-aminoimidazole-4-carboxamide ribotide (AICAR) transformylase (phosphoribosylaminoimidazolecarboxamide formyltransferase, EC 2.1.2.3) originally developed by Flaks *et al.* (1957) and Flaks & Lukens (1963) has the following limitations: (1) the colour of the azo dye of AICAR resulting from the Bratton–Marshall assay begins to fade within 2 h; (2) no antioxidant (e.g. mercaptoethanol) is included to protect the labile folate substrate, 10-formyltetrahydrofolate (10-HCO- H_4 folate) and labile folate product (H_4 folate); (3) since the assay measures the disappearance of the azo dye of AICAR, the measurement of a small difference in large absorbances is very susceptible to error.

A modified colorimetric assay is based upon the selective oxidation of H_4 folate to *p*-aminobenzoyl glutamate (pABG), described by Maruyama *et al.* (1978) and Eto & Krumdieck (1980). In this procedure, the Bratton–Marshall assay detects the azo dye of pABG and mercaptoethanol is added to protect labile folate coenzymes. The modified colorimetric assay allows the detection of inhibition of the transformylase by drugs which have a high absorbance at 312 nm, which is the wavelength used in the direct spectrophotometric assay (Baggott *et al.*, 1986).

It is now recognized that substances with anti-folate activity, such as methotrexate (MTX) and sulphasalazine, are useful in the treatment of autoimmune diseases such as rheumatoid arthritis. Azathioprine is also useful in the treatment of rheumatoid arthritis and is an immunosuppressant (Calabresi & Parks, 1985). We therefore decided to test whether azathioprine and its metabolite, thioinosinic acid (tIMP), also interfere with the folate-dependent enzyme of purine biosynthesis, AICAR transformylase.

EXPERIMENTAL

Materials

Chicken liver AICAR transformylase was purified as described previously (Baggott *et al.*, 1986). 5,10-Methenyltetrahydrofolate (5,10-CH⁺- H_4 folate) was prepared by the method of Rabinowitz (1963), and stock solutions in 5 mM-HCl/10 mM-mercaptoethanol were stored at $-20^\circ C$. 5,10-CH⁺- H_4 folate was converted into 10-formyltetrahydrofolate (10-HCO- H_4 folate) by adjusting the pH to 7.4 with phosphate buffer. The folate coenzyme and AICAR were quantified as previously described (Baggott & Krumdieck, 1979).

tIMP and azathioprine were purchased from Sigma Chemical Co. All other reagents were the best grade available from commercial sources. 10-Deaza-aminopterin (DAAM) was a gift from Dr. Gopal Nair (University of South Alabama, Mobile, AL, U.S.A.).

MRL/lpr mice were originally obtained from Jackson Laboratories (Bar Harbor, ME, U.S.A.) and a breeding colony was established. These female mice develop a systemic autoimmune disease (Theofilopoulos & Dixon, 1981).

MRL/lpr mice were treated from 7 weeks of age until they were killed (30 weeks of age) with the following drug protocols: 5, 25 and 100 mg of MTX/week per kg body wt. (designated M1, M2, M3 groups respectively), and 1 or 25 mg of DAAM/week per kg body wt. (designated D1 and D2 groups respectively). All drugs were dissolved in phosphate-buffered saline (0.01 M-phosphate/0.13 M-NaCl, pH 7.2) and injected intraperitoneally twice each week. These experiments were carried out to test the effect of MTX and DAAM on the systemic autoimmune disease.

Mouse and human peripheral-blood mononuclear cells (PBMCs) were isolated from heparinized whole blood by

Abbreviations used: AICAR, 5-aminoimidazole-4-carboxamide ribotide; H_4 folate, tetrahydrofolate; pABG, *p*-aminobenzoyl glutamate; PBMC, peripheral-blood mononuclear cells; tIMP, thioinosinic acid; MTX, methotrexate; HCO-, formyl; CH⁺-, methenyl; DAAM, 10-deaza-aminopterin; 6-MP, 6-mercaptopurine.

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continuous density-gradient centrifugation in Sepracell-MN (Sepratech Corp., Oklahoma City, OK, U.S.A.). PBMCs were washed and suspended in Krebs–Ringer bicarbonate buffer and quantified by using a haemocytometer. Cell extracts were made by freezing and thawing the suspension (three times), followed by centrifugation to remove insoluble cell debris. The DNA content of cell suspensions was determined by the method of Kissane & Robins (1958).

Methods

A capped plastic assay tube was used and contained (final concentrations) 50 mM-potassium phosphate buffer, pH 7.4, 10 mM-2-mercaptoethanol, 20–100 μ M-10-HCO-H₄folate, 20–100 μ M-AICAR and the enzyme preparation, in a total volume of 1 ml. The assay was initiated by the addition of the enzyme preparation immediately before placing it in a 37 °C water bath.

Tubes were incubated for 1–2 h, and the enzyme-catalysed reaction was terminated by the addition of 0.1 ml of 0.17 M-HgCl₂ and 0.1 ml of 1 M-ammonium bicarbonate, pH 9.2. After mixing, 0.2 ml of 2% (w/v) potassium permanganate was added (with mixing) and the suspension was incubated at room temperature. After a 5 min incubation, 0.8 ml of 0.3% H₂O₂ solution was added and the tubes were centrifuged at 2000 *g* for 5 min. A portion (1 ml) of the supernatant was pipetted to a test tube in an ice bath and 0.1 ml of 3 M-NaCl, 0.6 ml of 5 M-H₂SO₄ and 0.2 ml 1% (w/v) NaNO₂ were added with mixing. After a 3 min incubation on ice, 0.2 ml of 5% (w/v) ammonium sulphamate was added (with mixing). After a 1 min incubation on ice, 0.2 ml of 1.5% (w/v) *N*-(1-naphthyl)ethylenediamine was added (with mixing). The colour was allowed to develop at room temperature for 2 h and the absorbance at 560 nm was measured.

Colour blanks for the assay contained all of the assay components except the enzyme preparation, or AICAR was added to all of the other components of the assay just before the termination of the enzyme-catalysed reaction. A standard curve is obtained by substituting pABG (0 to 100 μ M) in place of 10-HCO-H₄folate and using the highest AICAR concentration. The amount of H₄folate formed during the incubation and subsequently oxidized to pABG could be determined from the standard curve. When an inhibitor was used, a standard curve was determined in the presence of the highest concentration of the inhibitor. This curve was used to determine the amount of pABG formed in experiments using the highest inhibitor concentration. The amount of pABG formed in experiments using an intermediate inhibitor concentration was estimated by interpolation.

The HgCl₂, potassium permanganate, NaCl and H₂SO₄ solutions are stable and were stored in sealed bottles at room temperature. The ammonium bicarbonate, sodium nitrite, and *N*-(1-naphthyl)ethylenediamine solutions were stored in sealed bottles at 5 °C and were freshly prepared every 2 months.

Initial velocities and corresponding substrate concentrations (of the limiting substrate) were calculated by the method of Waley (1981). Kinetic parameters and inhibition constants were estimated by using the computer programs described by Cleland (1979).

RESULTS AND DISCUSSION

Chemical rationale of the assay

The assay is based upon the observation that H₄folate is oxidatively cleaved to pABG by alkaline permanganate treatment, whereas 10-HCO-H₄folate is oxidized primarily to 10-HCO-folate (Maruyama *et al.*, 1978; Eto & Krumdieck, 1980). Thus pABG may be detected by the Bratton–Marshall assay,

whereas the oxidation products of 10-HCO-H₄folate after alkaline permanganate treatment are not detected by this assay.

Whereas pABG is relatively stable to short-term alkaline permanganate oxidation (Maruyama *et al.*, 1978; Eto & Krumdieck, 1980), our results show that AICAR is not stable to this oxidation. In experiments without pABG, the 5 min alkaline permanganate oxidation followed by the Bratton–Marshall assay yields a colourless solution when AICAR is present at an initial concentration of 500 μ M. In contrast, the standard curves of pABG with or without the alkaline permanganate oxidation (i.e. water substituted in the potassium permanganate and H₂O₂ additions) shows only a 1–2% difference in the colour yield.

Advantages of the modified assay

Meraptoethanol is included in the new assay to protect the labile substrate 10-HCO-H₄folate and the labile product H₄folate from air oxidation. The oxidation products of the above compounds (i.e. 10-HCO-H₄folate, 10-HCO-folate, H₄folate or folic acid) are either poor substrates for the transformylase or inhibitors (Baggott *et al.*, 1986).

The azo dye obtained in the Bratton–Marshall assay of pABG is stable for 24 h in contrast with the azo dye obtained in the Bratton–Marshall assay of AICAR. Our results show that the colour obtained with AICAR begins to fade 2 h after the *N*-(1-naphthyl)ethylenediamine reagent is added.

We have found that other assays of the transformylase (Flaks & Lukens, 1963; Baggott & Krumdieck, 1979; Baggott *et al.*, 1986) are not suitable for detecting low activity in crude extracts of some tissues. This agrees with a similar observation by Erbe (1979). In contrast, the new assay can be used with crude extracts of tissues such as PBMCs from the MRL/lpr mouse. Table 1 demonstrates that the coefficient of variation for a single sample of PBMCs ranges from approx. 10–25% of the mean using three or four levels of enzyme.

The mean ($\pm$ S.D.) activity of the transformylase in extracts of PBMCs of nine MRL/lpr mice was 1.98 ± 0.92 (μ M of pABG/h) per μ g of DNA. The mean ($\pm$ S.D.) activity of the transformylase in extracts of PBMCs from six humans was 1.25 ± 0.67 μ M-pABG/h per 1×10^6 cells. The above coefficients of variation are greater than that observed for the transformylase measured in rat livers (Deacon *et al.*, 1985). However, liver is a much richer source of the enzyme and variations in the specific activity may be smaller.

Finally, in a non-continuous colorimetric assay, it is better to measure the appearance of a coloured product (i.e. an azo dye of pABG) rather than the disappearance of a coloured substrate

Table 1. Activity of AICAR transformylase in extracts of MRL/lpr mouse PBMCs

Treatments are described in the Experimental section. Pooled blood from two or three animals was used to isolate PBMCs; results are means $\pm$ S.D., and *n* is the number of assays done. Assays were performed using three or four levels of enzyme, with replication, using 100 μ M-10-HCO-H₄folate and 100 μ M-AICAR as described under 'Methods'.

Treatment	Activity (μ M-pABG/h per 10^6 cells)
M1	2.70 ± 0.56 (8)
M2	3.81 ± 0.94 (6)
M3	4.27 ± 0.86 (6)
D1	5.30 ± 0.76 (8)
D2	10.26 ± 1.25 (6)

(i.e. an azo dye of AICAR). The latter case requires the measurement of small differences in large absorbances and is more susceptible to error.

Inhibition of AICAR transformylase by tIMP, azathioprine and other drugs

Both azathioprine and its metabolite, tIMP, have high absorbances at 312 nm, which precludes the use of the continuous and direct spectrophotometric assay of the transformylase (Baggott *et al.*, 1986) to detect inhibition by these drugs. However, neither tIMP nor azathioprine, at a concentration of 484 μM , substantially affected the standard curve of pABG in our modified assay.

As Figs. 1 and 2 show, both tIMP and azathioprine are competitive inhibitors (with respect to AICAR) of the chicken liver enzyme and the enzyme from PBMCs of the MRL/lpr mouse. The K_i values of tIMP and azathioprine for the chicken liver enzyme are $39 \pm 4 \mu\text{M}$ and $120 \pm 10 \mu\text{M}$, whereas the K_i values for the enzyme from PBMCs of the MRL mouse are $110 \pm 20 \mu\text{M}$ and $90 \pm 14 \mu\text{M}$ respectively. Interestingly, the K_i of tIMP is

Fig. 1. Competitive inhibition of chicken liver AICAR transformylase by tIMP (a) and azathioprine (b)

The reciprocal of the initial velocity ($1/v$, in arbitrary units) is plotted against the reciprocal of the corresponding concentration of AICAR ($1/[AICAR]_{\text{corresponding}}$). The initial 10-HCO- H_4 folate concentration was 100 μM ; the tIMP (a) concentration was 0 μM ($\circ$), 121 μM ($\bullet$), 242 μM ($\triangle$) or 484 μM ($\square$), and the azathioprine (b) concentration was 0 μM ($\circ$), 242 μM ($\triangle$) or 484 μM ($\square$). All other conditions are as described under 'Methods'.

Fig. 2. Competitive inhibition of mouse PBMC AICAR transformylase by tIMP (a) and azathioprine (b)

The reciprocal of the initial velocity (in arbitrary units) is plotted against the reciprocal of the corresponding AICAR concentration (as in Fig. 1). The initial 10-HCO- H_4 folate concentration was 100 μM , and the initial tIMP and azathioprine concentrations were 0 μM ($\circ$), 242 μM ($\bullet$) and 484 μM ($\square$). All other conditions are as described under 'Methods'.

somewhat lower than that of azathioprine in chicken liver, but no substantial difference in the K_i values was found in the mouse PBMC enzyme. This suggests that there may be substantial differences in the enzymes from these two sources. However, the apparent K_m values for AICAR varied only from 12 to 16 μM (at 100 μM ; 10-HCO- H_4 folate), suggesting that both chicken liver and mouse PBMC enzymes have essentially the same affinity for this substrate. Since the free base and riboside of AICAR are not substrates for the transformylase (Flaks *et al.*, 1957), inhibition of the enzyme by azathioprine was an unexpected finding.

It is interesting that tIMP is also a competitive inhibitor of adenylosuccinate lyase (Atkinson *et al.*, 1964), which is a bifunctional enzyme of purine metabolism catalysing the cleavage of both adenylosuccinate and 5'-phosphoribosyl-4-(*N*-succinocarboxamide)-5-aminoimidazole to AMP and AICAR respectively (Woodward, 1978). These results are consistent with a structural similarity of tIMP and AICAR which allow tIMP to compete with AICAR at the active sites of other enzymes (i.e. the transformylase).

By using the modified assay, the inhibition of the transformylase could be tested with a ratio of tIMP or azathioprine concentration to AICAR concentration of up to 48. This high ratio is likely to occur *in vivo*. Odland *et al.* (1986) found that tIMP concentration in red blood corpuscles is approx. 2 μM after a 100 mg intravenous infusion of azathioprine, whereas the AICAR concentration is too low to be detected in tissues and tissue homogenates which are synthesizing purine nucleotides *de novo* (Sabina *et al.*, 1982). In addition, AICAR concentration is too low to be detected in partially purified enzyme systems

Table 2. Percentage inhibition of chicken liver AICAR transformylase by drugs used in the treatment of autoimmune diseases

Drug	Concn.	Assay conditions...	Inhibition (%)	
			100 μ M-10-HCO-H ₄ folate + 100 μ M-AICAR	100 μ M-10-HCO-H ₄ folate + 20 μ M-AICAR
Naproxen	2 mM		-46*	-32
Ibuprofen	2 mM		-22	-14
4-Acetamidophenol	500 μ M		-6	0
Chloroquine	484 μ M		+1	-1
tIMP	484 μ M		-53	
Azathioprine	484 μ M		-43	

* A minus sign means inhibition of enzyme activity by the drug.

from avian liver and human lymphocytes, which are actively biosynthesizing purine nucleotides (Rowe *et al.*, 1978; McCairns *et al.*, 1983). In cell cultures, Bokkerink *et al.* (1986) detected an intracellular AICAR concentration of approx. 1–5 μ M in exponential-phase cultures of malignant human lymphoblasts. By contrast, the intracellular tIMP concentration was approx. 200–300 μ M in the cells exposed to an extracellular concentration of 6-mercaptopurine (6-MP) of 10 μ M. The latter extracellular concentration is not physiologically unrealistic, since peak plasma concentrations of 6-MP are 1–3 μ M after intravenous or oral administration of azathioprine or 6-MP (Odlind *et al.*, 1986; Whalen *et al.*, 1985).

The above results suggested that other drugs which are used in autoimmune diseases (especially rheumatoid arthritis) could also be inhibitors of the transformylase. Table 2 shows that naproxen and ibuprofen are also inhibitors of the transformylase from chicken liver, whereas 4-acetamidophenol (i.e. paracetamol, tylenol) and chloroquine are not. It is interesting that 4-acetamidophenol, which has little or no anti-inflammatory activity, is not an inhibitor, whereas those drugs with anti-inflammatory activity (i.e. naproxen and ibuprofen) do inhibit the enzyme. MTX and sulphasalazine, which are useful in rheumatoid arthritis and other autoimmune diseases, are also known to have anti-folate activity (Weinblatt *et al.*, 1985; Pullar *et al.*, 1985). MTX inhibits dihydrofolate reductase, AICAR transformylase and a number of other folate-dependent reactions (Baggott *et al.*, 1986; Chabner *et al.*, 1986). Sulphasalazine has also been shown to inhibit folate-dependent enzymes *in vitro* and in cultured lymphocytes (Selhub *et al.*, 1978; Halsted, 1980; Baum *et al.*, 1981). Interference of folate metabolism may be a common attribute of many anti-inflammatory drugs.

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