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INHIBITION OF HUMAN ERYTHROCYTE OROTIDYLATE DECARBOXYLASE

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Abstract—Ribonucleotide derivatives of allopurinol and oxipurinol are potent inhibitors of human erythrocyte orotidylate decarboxylase. The inhibition constants are dependent upon the aggregation state of the enzyme, much tighter binding being observed with higher molecular weight forms. The trend was similar to that observed for K_m values for orotidine-5'-phosphate, the substrate of the enzyme. Of the compounds tested, 1-oxipurinol-5'-phosphate, with a K_i value of 0.3 nM for the 250,000 M.W. species, was the most effective inhibitor. This was some two orders of magnitude tighter than 7-oxipurinol-5'-phosphate, which in turn was two orders of magnitude tighter than 1-allopurinol-5'-phosphate. A similar trend of K_i estimates with molecular weight of the enzyme was observed with a number of other inhibitors, including 3-XMP, 9-XMP, 6-azaUMP, UMP and inorganic phosphate (HPO_4^{2-}).

Orotidylate decarboxylase (ODCase; EC 4.1.1.23) converts orotidine-5'-monophosphate (OMP) to UMP in *de novo* pyrimidine biosynthesis. It is inhibited by its product and also is known to be inhibited by metabolites of a number of purine and pyrimidine analogs, notably 6-azauridine [1, 2] and the hypoxanthine analog, allopurinol (4-hydroxypyrazolo-(3,4-D)-pyrimidine [3, 4].

Metabolites of allopurinol, and of its major *in vivo* product, oxipurinol (4,6-dihydroxypyrazolo-(3,4-D)-pyrimidine), were first implicated as inhibitors of ODCase when administration of allopurinol was shown to cause orotic aciduria and orotidinuria in man [3, 4]. Work in this laboratory demonstrated potent inhibition of erythrocyte ODCase following preincubation of either allopurinol or oxipurinol with phosphoribosylpyrophosphate in the presence of haemolysate [3, 5] and Kelley and Beardmore [4] reported that 1-allopurinol ribonucleotide was a competitive inhibitor of this enzyme with a K_i of 0.8 μM.

The metabolic fate of allopurinol has been studied most extensively in the rat [6, 7] where a number of derivatives were found, in liver, in the nanomole to micromole range, including 1-allopurinol-5'-phosphate, 1-oxipurinol-5'-phosphate and 7-oxipurinol-5'-phosphate. In the red cell, the level of allopurinol ribonucleotide was ~10 per cent of that found in the liver and no oxipurinol ribonucleotides were detected. Synthesis of these ribonucleotides was achieved and inhibition of ODCase from yeast and liver demonstrated [8].

The current study was undertaken in order to determine accurate inhibition constants of the different molecular species of ODCase from human erythrocytes by the synthetic allopurinol metabolites. We have demonstrated that orotidylate decarboxylase from human erythrocytes usually copurifies as a complex with the preceding enzyme in the pathway, orotate phosphoribosyl-transferase (EC 2.4.2.10) and that the complex can exist in three forms with molecular weights corresponding approximately to 62,000, 115,000 and 250,000 [9]. The three forms, corresponding to monomer, dimer and tetramer, could be separated by chromatography on Sephadex G-200 and maintained their integrity for 3–6 days. As the different oligomers were found to differ both in stability and kinetic properties, it was of interest to determine whether this was reflected in the degree of inhibition by the various compounds. A number of other nucleotides were also tested as inhibitors and these results are included.

MATERIALS AND METHODS

Materials. [*Carboxyl*-^{14}C]OMP was purchased from New England Nuclear Corp. OMP, UMP, XMP, XDP, XTP, UDP, TMP, CMP, CDP, GMP, GDP, GTP, AMP, ADP, ATP, ITP and 6-azaUMP were all obtained from either Sigma Chemical Co. or Calbiochem Pty. Ltd.

1-Allopurinol ribonucleotide, 1-oxipurinol ribonucleotide, 7-oxipurinol ribonucleotide and 3-XMP were gifts from Dr. J. A. Fyfe, Burroughs Wellcome & Co. (U.S.A.) Inc., 3030 Cornwallis Road, Research Triangle Park, NC 27709, U.S.A. DEAE cellulose was obtained from Whatman Biochemicals Ltd. and Sephadex G-150 and G-200 from Pharmacia Fine Chemicals.

Enzyme assay. Orotidylate decarboxylase was assayed by following the release of $^{14}CO_2$ from [*carboxyl*-^{14}C]OMP [5, 9]. The usual reaction mixture contained [*carboxyl*-^{14}C]OMP, 0.08 mM (7 nCi), Tris–HCl, pH 7.4, 50 mM, and enzyme in a final volume of 1.0 ml. Enzyme activity was expressed as nmoles CO_2 produced/mg protein/hr. Protein concentration was determined using the method of Goodwin and Choi [10].

Enzyme. Orotidylate decarboxylase was partially purified from human erythrocytes as previously de-

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scribed [9]. The resulting preparation consisted of a tightly bound complex of the decarboxylase and orotate phosphoribosyltransferase and was resolved into three molecular weight forms by chromatography on a 60 × 1.5 cm column of Sephadex G-200 in 50 mM potassium phosphate, pH 7.4. Fractions containing the three peaks of activity were separately pooled, concentrated by suction in collodion bags (Sartorium Membrane filter SM 13200), and stored in the elution buffer.

RESULTS

Allopurinol derivatives. Because of limited supplies of these compounds, studies with the allopurinol and oxipurinol derivatives were principally confined to the 115,000 and 250,000 molecular weight forms of ODCase. In general, all were very good competitive inhibitors of the enzyme, tighter binding being observed with the higher molecular weight species as might have been anticipated from the lower K_m values reported [9]. The estimates are collected in Table 1 together with equivalent values obtained by Fyfe *et al.* [8] for ODCase from yeast and rat liver and with respective K_m values from this laboratory [9]. The compound, 1-allopurinol-5'-phosphate, was available in greater quantity and could also be tested with the 62,000 molecular weight form.

Inhibition constants for a number of other competitive inhibitors of the enzyme are included in Table 1. These included the synthetic analog, 3-XMP, 9-XMP, 6-azaUMP and the product of the reaction, UMP. As found for plots of velocity with respect to substrate [9], apparent biphasic or triphasic plots could be observed for the inhibition studies with mixtures of two or three molecular weight forms of the enzyme. In general, the inhibition constants are consistent with values obtained for ODCase by other workers and from other sources. However, even the K_i values for the higher molecular weight form of the enzyme are somewhat higher (by a factor of 2–3) for allopurinol-5'-phosphate and 9-XMP than the values of 0.8 μM and 0.7 μM respectively, reported by Kelley and Beardmore [4].

Other nucleotides. A number of other nucleotides were tested as inhibitors of two molecular weight forms, 250,000 and 62,000, of erythrocyte ODCase. The concentration of the substrate was 0.5 and 20 μM respectively, and of the potential inhibitors, 5 μM and 0.5 μM respectively. These latter values corresponded to the concentration of 9-XMP required for 50 per cent inhibition at these substrate concentrations.

With the 250,000 molecular weight form, a slight degree of inhibition was seen with UDP (12 per cent) and XDP (10 per cent). Less than 10 per cent inhibition was seen with the other nucleotides tested, viz, AMP, ADP, ATP, GMP, GDP, GTP, XTP, ITP, CMP, CDP, TMP. Similarly, for the 62,000 molecular weight form, moderate inhibition was seen with XDP (25 per cent) and slight inhibition with UDP (15 per cent) and GMP (12 per cent). Less than 10 per cent inhibition was seen with the remainder of the same range of compounds as above. It is appreciated that more rigorous experiments could have found conditions in which many of the nucleotides did act as effective inhibitors but these experiments were not pursued.

Effect of phosphate. In a previous study, inhibition of human erythrocyte ODCase by inorganic phosphate was demonstrated, although the K_i value was very high (~0.9 M) [9]. This effect of phosphate could influence some of the kinetic results as the enzyme was routinely stored in 50 mM potassium phosphate, pH 7.4. Studies of phosphate inhibition of the isolated molecular weight forms demonstrated that the high K_i value only applied to the 62,000 and 115,000 forms. The 250,000 molecular weight form was considerably more sensitive to phosphate inhibition with a K_i of ~22 mM. The concentration of phosphate in the usual assay mixture would therefore be suffi-

Table 1. Inhibition of orotidylate decarboxylase. Comparison of K_i values for several allopurinol metabolites and other inhibitors of orotidylate decarboxylase compared to the relevant K_m values

	Erythrocyte			Yeast*		Rat liver*	
	250,000	115,000	62,000	Low OMP	High OMP	Low OMP	High OMP
1-allopurinol-5'-phosphate	2.6	8.0	70	1.0	3.0	1.0	3.0
1-oxipurinol-5'-phosphate	0.0003	0.004		0.003	0.02	0.0005	0.002
7-oxipurinol-5'-phosphate	0.06	0.8		0.06	0.7	0.04	0.2
3-XMP	0.15	0.45		0.3	1.0	—	—
9-XMP	1.9	6.3	90	1.0	10.0	—	—
6-azaUMP	0.06	0.12	0.6	0.7†		0.1‡	
UMP	11	88	2600	400‡		150‡	
OMP§	0.6 ± 0.25	3.3 ± 0.6	25	0.45 ± 0.14	2.4 ± 0.7	1 ± 0.3	4 ± 1

The K_i values were determined separately for the 250,000, 115,000 and 62,000 molecular weight forms and were derived from Dixon plots [11]. Substrate concentrations for the 250,000 molecular weight form were 0.5 and 1.0 μM, for the 115,000 molecular weight form, 5 and 10 μM, and for the 62,000 molecular weight form, 20 μM. The results are compared with the values obtained in yeast and rat liver at low and high OMP concentration by Fyfe *et al.* [8].

Values are all in μM.

* Fyfe *et al.* [8].

† Handschumacher [12].

‡ Creasey and Handschumacher [13].

§ Relevant K_m values.

cient to effect a small but measurable alteration in the estimates of kinetic constants for this form of the enzyme. This effect has been taken into account in calculations of the constants described in Table 1.

DISCUSSION

The three ribonucleotide derivatives of allopurinol and oxipurinol tested were all very potent inhibitors of erythrocyte ODCase, with 1-oxipurinol-5′-phosphate being the most effective. The inhibition constants followed the same trend (Table 1) with respect to the different molecular weight species of ODCase as was observed for the K_m values [9]. The results are similar to those obtained in studies of ODCase from yeast and rat liver. Presumably, 1-oxipurinol-5′-phosphate is formed *in vivo* by hypoxanthine-guanine phosphoribosyltransferase (HGPRTase) as oxipurinol is an analog of xanthine and thus of hypoxanthine. The extremely low K_i value of 0.3 nm for the 250,000 molecular weight species suggested that this compound may be of considerable importance in the inhibition of ODCase *in vivo*.

Studies of cells from patients with HGPRTase deficiency has also demonstrated inhibition of ODCase in the presence of allopurinol [5, 14], implying a further derivative of the drug, probably 7-oxipurinol-5′-phosphate, synthesized by an alternative phosphoribosyltransferase. This compound, which can be demonstrated in man following allopurinol administration [15], is probably formed by orotate phosphoribosyltransferase and is also a potent inhibitor of ODCase *in vitro*. The degree of inhibition of ODCase in HGPRTase deficient cells is less than in normal cells suggesting that both compounds contribute to the *in vivo* effects. It is noteworthy that allopurinol ribonucleotide is significantly less potent as an ODCase inhibitor *in vitro* than the oxipurinol derivatives, presumably reflecting the greater structural similarity of oxipurinol to orotic acid.

Inhibition of ODCase by 6-azaUMP is also a major *in vivo* effect of this drug. In a study of the yeast enzyme, the K_i for 6-azaUMP was determined as 0.7–0.8 μM [12], similar to the results obtained with the human erythrocyte enzyme, although the 250,000 molecular weight form is considerably more sensitive to inhibition by this compound, a K_i of 0.06 μM being observed. Inhibition of ODCase by 6-azaUMP does occur *in vivo* following administration of therapeutic doses. The inhibition is reflected in increased urinary excretion of orotic acid and orotidine and decreased release of $^{14}CO_2$ from [*carboxyl*-^{14}C] orotic acid [1, 2, 16]. Inhibition constants for 6-azaUMP have also been determined for the cow brain enzyme (K_i, 0.4 μM) [17] and the rat liver enzyme (K_i, 0.1 μM) [13].

Of the naturally occurring nucleotides, UMP is the only one which has been consistently demonstrated as an inhibitor of the enzyme ODCase from various tissues [4, 13, 17, 18]. In all studies, the K_i for UMP has been relatively high, viz, 0.25 mM in cow brain [17], 0.4 mM in yeast [13], 0.15 mM and 6.9 mM in rat liver [11, 18] and ~0.5 mM in hemolysate [4]. In the present study, values of this order were only obtained with the 62,000 molecular weight form and the two higher molecular weight forms were much more sensitive to UMP inhibition. 9-XMP was shown to be a potent inhibitor of the human erythrocyte enzyme by Kelley and Beardmore [4]. This inhibition has been confirmed in the present study. However, the K_i value of 0.7 μM reported previously was found to correspond to the 250,000 molecular weight form only, with the other forms being less sensitive.

Evidence for inhibition of ODCase by other naturally occurring nucleotides is not as clear. Inhibition of the cow brain enzyme [17] and yeast and rat liver enzymes [13] has been demonstrated with CMP, AMP and GMP but the K_i values were high, in the range 0.1–1.0 mM. The rat liver enzyme [12] appeared to be more specific. It was not inhibited by AMP or GMP and the K_i for CMP was much greater than that obtained for the yeast enzyme. Thus, the high K_i values for the naturally occurring nucleotides compared to the K_m value for OMP suggest that, in general, inhibition of this enzyme is not a major factor in *in vivo* regulation.

In conclusion, it is appreciated that studies of inhibition of human erythrocyte ODCase do not provide direct evidence for effects which occur *in vivo* as the pyrimidine biosynthetic pathway is incomplete in the mature red cell. However, the pattern of inhibition is very similar to that of the rat liver enzyme suggesting that the enzyme in erythrocytes does reflect properties of the enzyme in tissues which are active in pyrimidine biosynthesis. For this reason inhibition studies of the red cell enzyme provide indirect evidence for the mechanisms by which various drugs interfere with the *de novo* pyrimidine biosynthetic pathway.

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