INHIBITION OF 5,10-METHYLENETETRAHYDROFOLATE DEHYDROGENASE BY PURINE NUCLEOTIDES *

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A new type of regulation whereby an end product can control the level of a coenzyme which is required for formation of the end product has been described in the case of methionine biosynthesis. Methionine was shown to repress the formation of 5,10-methylenetetrahydrofolate reductase (Katzen and Buchanan, 1965). Such a control serves to conserve the pivotal methylene derivative by preventing its one-way drain into 5-methyltetrahydrofolate.

In the other direction, oxidation of 5, 10-methylenetetrahydrofolate provides the two forms that are used in the transformylation of intermediates in the biosynthesis of purine nucleotides (Buchanan and Hartman, 1959). The oxidizing enzyme, 5, 10-methylenetetrahydrofolate dehydrogenase, has been described in animal tissues (Jaenicke, 1955; Greenberg et al., 1955; Osborn and Huennekens, 1957; Yeh and Greenberg, 1965), in yeast (Ramasastri and Blakley, 1962) and in bacteria (Donaldson et al., 1965). We have studied this enzyme in Salmonella and this communication shows that purine nucleotides control the oxidation of 5,10-methylenetetrahydrofolate in a manner analogous to the control of its reduction by methionine.

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MATERIALS AND METHODS

Bacteria used were the wild type Salmonella typhimurium, strain LT-2 and two of its purine requiring mutants, pur E-11 and pur B-12.

Aerated cultures were grown for 18 hours in a minimal salts-glucose medium, with additions as indicated. The cells were harvested, washed twice with saline, suspended in 0.01 M potassium phosphate buffer (pH 7.5) containing 2-mercaptoethanol (2 mM), ruptured by a MSE ultrasonic disintegrator and centrifuged at 30,000 x g for 1 hour. The clear extract was used as the enzyme source.

The assay of 5,10-methylenetetrahydrofolate dehydrogenase was based on the spectrophotometric measurement of the reduction of $NADP^+$. It was essentially the same as that used by Ramasastri and Blakley (1962) except that 2-mercaptoethanol, 10 mM, was substituted for dimercaptoethanol and the reaction was carried out at room temperature. A small amount of endogenous activity was observed with the crude extracts in the absence of formaldehyde. Activity in the absence of tetrahydrofolate or $NADP^+$ was negligible. The enzyme unit is defined as that amount required to produce a change in absorbancy at 340 mμ of 0.01 per minute. Specific activity is represented as units of enzyme per mg of protein in the extract. Only the initial linear rate over the first 30 - 40 seconds was used as a measure of enzyme activity. This period obviated the ensuing decrease in rate, presumably due to the subsequent oxidation of NADPH formed.

RESULTS

A comparison of the inhibitory effect of adenine and its derivatives on the 5,10-methylenetetrahydrofolate dehydrogenase of S. typhimurium strain LT-2 is given in Table I. Neither adenine nor adenosine were inhibitory up to concentrations of 4 mM, the maximum tested, while at the same concentration, AMP gave 58.5% inhibition. ATP was by far the most potent, a concentration of 0.5 mM producing 72.5% inhibition.

TABLE I

Inhibition of 5,10-methylenetetrahydrofolate dehydrogenase by adenine and its derivatives

Additions	Conc. mM	Specific activity	% Inhibition
None	–	2.9	
Adenine	4.0	2.9	0
Adenosine	4.0	2.8	0.5
AMP	4.0	1.2	58.5
ATP	0.5	0.8	72.5

The reaction mixture contained formaldehyde, 0.33 mM; dl,L-tetrahydrofolic acid, 0.2 mM; $NADP^+$, 0.2 mM; 2-mercaptoethanol, 10 mM, and cell free extract in a total volume of 3.0 ml of potassium phosphate buffer, 50 mM, at pH 7.5.

Table II compares inhibition by various purine nucleotides, giving the amounts required to exert 50% inhibition of activity. The triphosphates were about ten times as

TABLE II

Concentration of purine nucleotides required for 50 per cent inhibition of 5,10-methylenetetrahydrofolate dehydrogenase

Addition	Concentration for 50% inhibition of activity (mM)
ATP	0.30
ITP	0.40
GTP	0.25
GDP	0.45
GMP	4.0
AMP	3.5
IMP	3.5

active as the monophosphates in all cases. GDP was slightly less effective than GTP. The nucleosides, adenosine, guanosine and inosine, were inactive up to a concentration of 4 mM, the maximum tested.

Results in Table III indicate that the combined effect of ATP and GTP is additive. However, ITP seems to show some synergism with both these nucleotides.

TABLE III

Combined effect of purine nucleotides on 5,10-methylenetetrahydrofolate dehydrogenase

Additions (0.15 mM)	Activity (%)	Theoretical Additive Activity (%)	Degree of Synergism (theoretical/actual)
None	100		
ATP	68		
GTP	68		
ITP	76		
ATP + GTP	45	46	1.02
ATP + ITP	45	52	1.13
GTP + ITP	39	52	1.33
ATP + GTP + ITP	21	35	1.67

No repression of 5,10-methylenetetrahydrofolate dehydrogenase activity was observed when S. typhimurium strain LT-2 was grown in adenine (50 µg/ml)(Table IV). It was possible that the enzyme was already maximally repressed by the endogenous pool of purine nucleotides and hence psicofuranine and 6,diazo-5,oxo-L-norleucine (DON), known inhibitors of purine nucleotide biosynthesis, were used in an attempt to derepress enzyme formation. However, cells grown in psicofuranine at a concentration of 50 µg/ml (which inhibits growth by 76%), or in the glutamine analog, DON, at a concentration of 0.05 µg/ml (which inhibits growth by 35%), showed no difference in the specific activity of the enzyme when compared to cells grown in the absence of either inhibitor.

On the other hand, when a purine auxotroph of S. typhimurium, pur E-11 was grown either in limiting concentrations of xanthine, or in xanthosine which is known to be poorly transported into this organism, thus preventing build up of endogenous purine nucleotide pools, a 2 to 3 fold derepression of the enzyme was consistently obtained (Table IV). Similar results were obtained with another purine auxotroph, pur B-12, in which case the endogenous pools were limited by growing the cells either in growth limiting concentrations of adenine or in adenosine 3'-phosphate which is also poorly transported (Table IV).

TABLE IV

Derepression of 5,10-methylenetetrahydrofolate dehydrogenase in S. typhimurium

Strain	Additions in growth medium (μg/ml)	Growth control (%)	Specific Activity
LT-2	None	100	2.5
"	Adenine (50)	85	2.7
pur E-11	Xanthine (50)	100	2.4
"	Xanthine (5)	32	5.2
"	Xanthosine (50)	39	5.2
pur B-12	Adenine (50)	100	2.5
"	Adenine (5)	33	6.3
"	Adenosine 3'-phosphate (50)	85	5.0

DISCUSSION

5,10-Methylenetetrahydrofolate dehydrogenase catalyzes the conversion of the 5,10-methylene ($-CH_2$) derivative to the 5,10-methylidyne (-CH=) derivative, which in turn can be hydrolyzed to the 10-formyl (HCO-) derivative. Both the latter forms are at the oxidation state of formic acid and may serve as precursors for the two reactions of purine synthesis, the formylation of glycinamide ribonucleotide and 5-amino,4-imidazole carboxamide ribonucleotide respectively (Buchanan and Hartman, 1959). Under conditions of feedback inhibition where purine nucleotides inhibit the activity of 5'-phosphoribosylpyrophosphate amidotransferase (Nierlich and Magasanik, 1965), no formyl acceptors would be formed. Since the enzymatic dehydrogenation reaction has been shown to favour greatly the formation of 5,10-methylidynetetrahydrofolate (Yeh and Greenberg, 1965), inhibition of the dehydrogenase by purines would provide a means of preventing the unnecessary accumulation of tetrahydrofolate into a form from which it could not be recovered by transformylation. Such an effect would provide an economical means of maintaining tetrahydrofolate levels for other functions.

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