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SPECIFIC BINDING TO ADENYLOSUCCINATE SYNTHETASE OF ANALOGS OF INOSINIC ACID WITH NITROGEN, SULFUR, AND CARBON SUBSTITUTED FOR PHOSPHATE OXYGENS

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SUMMARY

1. Binding of the phosphate moiety of IMP to adenylosuccinate synthetase (IMP:L-aspartate ligase (GDP), EC 6.3.4.4) of *Escherichia coli* has been investigated with the aid of analogs of IMP in which one phosphate oxygen of IMP is replaced by another atom.

2. Inosine 5′-phosphorothiolate, 5′-mercapto-5′-deoxyinosine 5′-*S*-phosphate, 5′-amino-5′-deoxyinosine 5′-*N*-phosphate and 6′-deoxyhomoinosine 6′-phosphonic acid substituted for IMP as substrates of the synthetase; in the presence of saturating levels of GTP and aspartate their v_{max} values relative to IMP (v_{max} = 1.00) were 0.024, 0.066, 0.0023 and 0.035, respectively.

3. The above four analogs and also AMP and 6′-deoxyhomoadenosine 6′-phosphonic acid were competitive inhibitors of the synthetase with respect to IMP with enzyme–inhibitor dissociation constants of 140, 70, 320, 490, 32 and 280 μM, respectively. The dissociation constant of IMP is estimated from these data to be approx. 50 μM.

4. The enzyme–substrate dissociation constant of 5′-mercapto-5′-deoxyinosine 5′-*S*-phosphate together with data on its secondary phosphoryl pK_a and the relative tendency of oxygen and sulfur to form hydrogen bonds has been taken to indicate that IMP probably binds to the synthetase preferentially as its phosphodianion and that the oxygen at C-5′ of IMP does not make a major contribution to IMP binding.

5. It is suggested that steric properties in the region of the phosphate group of IMP may exert a profound influence on spatial relations between substrates and the active site.

Abbreviations: 5′-thio-IMP, 5′-mercapto-5′-deoxyinosine 5′-*S*-phosphate; 5′-amino-IMP, 5′-amino-5′-deoxyinosine 5′-*N*-phosphate; 5′-methylene-IMP, 6′-deoxyhomoinosine 6′-phosphonic acid.

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INTRODUCTION

We have previously reported[1] that the phosphate moiety of IMP is necessary for binding to the IMP site of adenylosuccinate synthetase (IMP:L-aspartate ligase (GDP), EC 6.3.4.4) and that replacement of one phosphate hydroxyl of IMP by hydrogen (to give inosine 5′-phosphite) abolishes affinity for the IMP site. This communication describes kinetic studies of the effect on specific binding of modifications at other positions of the phosphate moiety of IMP. The nucleotide analogs examined were inosine 5′-phosphorothioate (which possesses a C–O–P(S)$(OH)_2$ system), 5′-mercapto-5′-deoxyinosine 5′-*S*-phosphate (5′-thio-IMP) (C–S–P(O)$(OH)_2$ system), 5′-amino-5′-deoxyinosine 5′-*N*-phosphate (5′-amino-IMP) (C–NH–P(O)$(OH)_2$ system), 6′-deoxyhomoinosine 6′-phosphonic acid (5′-methylene-IMP) (C–CH_2–P(O)$(OH)_2$ system) and 6′-deoxyhomoadenosine 6′-phosphonic acid (5′-methylene-AMP). The IMP analogs are substrates for adenylosuccinase synthetase of *Escherichia coli* and competitive inhibitors with respect to IMP, from which their enzyme–substrate dissociation constants have been determined. The findings are compared with those of earlier studies[2] with IMP dehydrogenase and the sulfur- and nitrogen-containing analogs of IMP.

MATERIALS AND METHODS

Chemicals

5′-Methylene-AMP has been synthesized by JONES AND MOFFATT[3] and was kindly supplied by Dr. J. G. Moffatt. The IMP analogs with P–N and P–S bonds were synthesized by procedures described previously[2]. To prepare 5′-methylene-IMP, 240 units (0.4 ml suspension in 50% glycerol–0.5 M KCl) of AMP deaminase (Sigma Chemical Co; 1 unit deaminates 0.47 mμmole of AMP/min) was added to a solution of 5′-methylene-AMP (6 mg) in a mixture of 1.4 ml of 0.01 M 2-(*N*-morpholino)ethanesulfonic acid buffer (pH 6.4) and 0.2 ml of 1.5 M KCl. After 24 h at 25° the mixture showed no further decrease in absorbance at 265 mμ, and was accordingly heated in boiling water for 4 min and centrifuged to remove coagulated protein. The supernate possessed an absorption maximum at 248 mμ, as expected for an inosine derivative at pH 6.4, and with paper chromatography in saturated $(NH_4)_2SO_4$–0.1 M sodium acetate (pH 6)–isopropanol (79:19:2, vol.) it gave a single ultraviolet-absorbing spot with the same R_F (0.59; starting material, 0.35) as IMP.

Enzyme preparation and assay

Escherichia coli strain B (10 g of wet packed cells; obtained from the Grain Processing Corp., Muscatine, Iowa) was ground for about 5 min with alumina (25 g, 80–200 mesh, Fisher Scientific Co.) which had been previously mixed with 4 ml of water. No intact bacterial cells could then be detected by microscopic examination. Adenylosuccinate synthetase was extracted from the alumina paste and purified to the stage of low-pH Fraction II by the methods of LIEBERMAN[4]. The enzymatic conversion of IMP to adenylosuccinate was followed by measurement of the increase in absorbance at 280 mμ. A Cary model 15 spectrophotometer and 1-cm cells were employed. All assay mixtures had a final volume of 1 ml and contained 0.15 M glycine buffer (pH 8.0) and 5 mM $MgCl_2$. This concentration of $MgCl_2$ has been reported by

LIEBERMAN[4] to be optimal for adenylosuccinate formation in the presence of the glycine buffer. After addition of GTP, IMP and/or the IMP analogs, and finally the enzyme preparation, mixtures were equilibrated for 5 min in the spectrophotometer (25–27°) after which the reaction was started by the addition of L-aspartic acid. The subsequent changes in absorbance at 280 mμ were recorded for at least 10 min against a blank solution which lacked aspartic acid. The initial reaction rates were linear and were proportional to enzyme concentration with the volumes (10–100 μl) of enzyme preparation which were used; with 100 μl of enzyme and initial concentrations of 3 mM L-aspartate, 0.42 mM GTP, and 0.25 mM IMP the increase in absorbance at 280 mμ was 0.015/min.

RESULTS

The kinetics of adenylosuccinate synthetase with the IMP analogs as substrates or inhibitors was studied in the presence of levels of GTP (0.3 mM) and aspartate (3.0 mM) which were saturating under the following conditions. With 0.17 mM IMP (K_m = 20 μM) and 0.185 mM GTP in the standard assay buffer, maximum velocity was produced by 1.2 mM L-aspartate; with 5.5 mM aspartate the velocity was 8% less. With 0.17 mM IMP and 3 mM aspartate, maximal velocity was attained with 0.3 mM GTP. Previous determinations[4,5] of K_m values of aspartate and GTP have employed arbitrary levels of the nonvariable substrates.

The four IMP analogs were substrates of adenylosuccinate synthetase; the initial velocity data are given in Fig. 1. If aspartate or GTP was omitted, no absorbance changes at 280 mμ occurred. The reaction rate in the case of 5′-amino-IMP was too slow to permit reliable initial velocity data to be obtained. With inosine 5′-phosphorothioate as substrate, no increase in reaction velocity was observed when the concentrations of GTP and aspartate were simultaneously increased 2-fold over the values given in Fig. 1. Michaelis constants and maximum velocity values derived from Fig. 1 are given in Table I and include a minimum value for the relative v_{max} of 5′-amino-IMP obtained under the conditions of Fig. 1 with 0.71 mM of 5′-amino-IMP and 200 μl of the enzyme preparation. Since this level of 5′-amino-IMP is more than 20-fold greater than its K_i value, the minimum relative v_{max} reported is probably within 20% of the actual value.

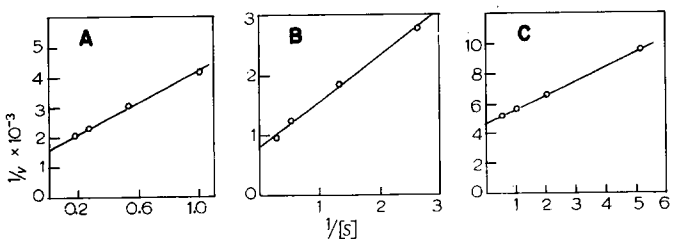

Fig. 1. IMP analogs as substrates of adenylosuccinate synthetase. Velocity is expressed as change in absorbance at 280 mμ per min and substrate concentrations are expressed as multiples of 10^{-4} M .Saturating levels of GTP (0.4 mM) and L-aspartate (3 mM) were employed; remaining assay conditions were as described in the text. A. 5′-Methylene-IMP (100 μl of enzyme preparation employed). B. 5′-Thio-IMP (100 μl of enzyme). C. Inosine 5′-phosphorothioate (50 μl of enzyme).

TABLE I

KINETIC CONSTANTS FOR SUBSTRATES AND INHIBITORS OF ADENYLOSUCCINATE SYNTHETASE

Compound	$K_i \times 10^5$ *(M)*	$K_m \times 10^5$ *(M)*	*Relative* v_{max}
IMP	—	2.0	1.00
5′-Methylene-IMP	49	16.4	0.035
5′-Thio-IMP	7.0	8.7	0.066
5′-Amino-IMP	32	—	0.0023*
Inosine 5′-phosphoro-thioate	14	2.0	0.024
AMP	3.2	—	—
5′-Methylene-AMP	28	—	—

* This is a minimum value for relative v_{max}; for explanation see under RESULTS.

Because of the low maximum velocities with the IMP analogs, it was possible to study them as inhibitors of the enzymatic conversion of IMP. Fig. 2 shows that inhibition by the IMP analogs as well as by AMP and 5′-methylene-AMP was in every case competitive with respect to IMP. Competitive inhibition by AMP with this enzyme has been previously reported[5]. The enzyme–inhibitor dissociation constants are given in Table I. These values were derived from secondary plots from Fig. 2 of inhibitor concentration against slopes which were linear in all cases.

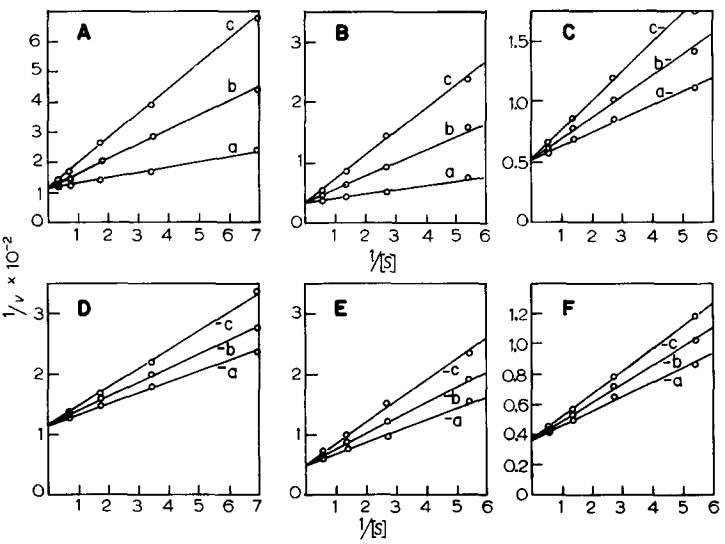

Fig. 2. Inhibition of adenylosuccinate synthetase. The reciprocal of initial velocity (change in absorbance per min) is plotted against one-tenth the reciprocal of the concentration (mM) of IMP. A. Inhibition by AMP, 0 mM (a), 42 μM (b), and 84 μM (c). B, Inhibition by 5′-thio-IMP, 0 mM (a), 152 μM (b), and 285 μM (c). C. Inhibition by inosine 5′-phosphorothioate, 0 μM (a) 85 μM (b), and 170 μM (c). D. Inhibition by 5′-methylene-AMP, 0 mM (a), 81 μM (b), and 162 μM (c). E. Inhibition by 5′-amino-IMP, 0 mM (a), 142 μM (b), and 284 μM (c). F. Inhibition by 5′-methylene-IMP, 0 mM (a), 153 μM (b), and 286 μM (c).

DISCUSSION

Our earlier studies[1] have shown that structural requirements for specific binding of IMP to adenylosuccinate synthetase of *E. coli* are complex. The 5′-phosphate moiety of IMP is essential because inosine does not bind to the IMP site as judged by its inability to act as a competitive inhibitor of IMP. The phosphoester bond of IMP is also crucial for binding inasmuch as neither P_i alone nor a combination of inosine and P_i is competitive with IMP. In addition, the hypoxanthine portion of IMP is required for specific binding because ribose 5-phosphate, at high concentration, displays no affinity for the IMP site. Replacement of a single ionizable hydroxyl of IMP by hydrogen (to give inosine 5′-phosphite) also prevents binding to the IMP site. Since this phosphite exists at the pH of the enzyme assay entirely as a phosphomonoanion and cannot form a phosphodianion, it was concluded[1] that either IMP is bound exclusively as its phosphodianion or, alternatively, that the hydrogen of the second ionizable hydroxyl of the phosphate of IMP forms a bond with the enzyme which is mandatory for specific binding.

In the present studies, the dissociation constant of IMP was not measured directly but it inferred to be approx. 50 μM, *i.e.* one-tenth the value for 5′-methylene-IMP in view of the finding (Table I) that the dissociation constant of AMP is one-tenth that of 5′-methylene-AMP. The observation that 5′-thio-IMP binds to the synthetase almost as well as IMP itself suggests that the esterified C-5′ oxygen of the phosphoester moiety of IMP does not make a major contribution to the total binding energy of IMP by hydrogen-bonding to the enzyme. This conclusion follows from the expectation that sulfur should hydrogen-bond less readily than the more electronegative oxygen; recent kinetic and thermodynamic studies[6] quantitatively confirm this relatively poor hydrogen-bonding ability of sulfur, *e.g.*, the heat of dimerization of 2-pyridone is 30% more negative than that of 2-thiopyridine. From this, and from the probable error (15%) in the K_i determinations, it is calculated that the energy of a hypothetical enzyme–H.....O(5′) bond would be less than 1.4 kcal/mole because substitution of sulfur for oxygen would then increase K_i by a factor (2.2) significantly greater than that observed.

At the pH (8.0) of the K_i determinations, 2.0% of IMP (secondary phosphate $pK_a = 6.3$) exists as a phosphomonoanion ($ROPO_3H^-$), whereas only 0.5% of 5′-thio-IMP ($pK_a = 5.7$; ref. 2) is monoanionic. The K_i value of 5′-thio-IMP tends to support the dianion of IMP as the preferred species inasmuch as monoanion binding would imply that 5′-thio-IMP binds approx. 4-fold better than IMP despite the increased bulk (S, 1.70 Å; O, 1.35 Å) at C-5′ and the altered phosphoester bond angle (*cf.* C–S–C, 104°; C–O–C, 110°) occasioned by replacement of the 5′-oxygen by sulfur. Earlier studies[2] with the present IMP analogs indicated that IMP dehydrogenase of *Aerobacter aerogenes* likewise appears to bind IMP as its dianion and that the 5′-oxygen is not strongly enzyme-bonded during IMP adsorption.

Replacement of the 5′-oxygen of IMP by methylene (van der Waals radius, 2.0 A) increases substrate bulk while presumably not changing the bond angle more than several degrees. By analogy with ethyl phosphonic acid ($pK_a = 7.85$; ref. 7) approx. 60% of 5′-methylene-IMP is dianionic at pH 8.0. If it is assumed that the dianionic species is bound selectively and without interference from the monoanionic species, the enzyme–substrate dissociation constant for 5′-methylene-IMP could be

expected to be approximately twice that of IMP because IMP is 98% dianionic at pH 8.0. The observed value was actually 10 times that of IMP and in view of the above conclusion that the 5′-oxygen of IMP is not involved in strong hydrogen-bonding to the enzyme, the substantial decrease in binding caused by the methylene group may tentatively be ascribed to steric interference.

5′-Amino-IMP, which probably exists at pH 8.0 principally as a dianionic zwitterion, $NH_2^+PO_3^{2-}$ (for discussion, see ref. 2) has a 6-fold larger enzyme–inhibitor dissociation constant than IMP. This, in similar fashion to 5′-methylene-IMP, could result from limited bulk tolerance at the 5′-oxygen of IMP; in addition, repulsive forces might operate between the positive nitrogen of 5′-amino-IMP and positive centers at the enzymic phosphoester binding site. Inosine 5′-phosphorothioate also binds less strongly than IMP. This phosphoester ($pK_a = 5.2$; ref. 2) is 99.8% dianionic at pH 8.0. It is not clear to what extent the diminished binding is ascribable to steric considerations, to the lesser hydrogen-bonding potential of sulfur, or to an imbalance in the symmetry of distribution of negative charge around phosphorus in the IMP dianion. The relative binding ability of the two sulfur-containing IMP analogs for the IMP site of IMP dehydrogenase of *Aerobacter aerogenes* resembles that reported here for the synthetase.

Table I shows that v_{max} for the IMP analogs varied from approx. 0.3 to 6.6% that for IMP; by contrast, with IMP dehydrogenase the lowest v_{max} for these analogs was 67% that of IMP[2]. In the case of inosine 5′-phosphorothioate it was established that the v_{max} observed was not an apparent value associated with increased Michaelis constants for GTP and/or aspartate, and the same is presumably true for the remaining analogs since they were studied with GTP in 25% excess over saturation and with aspartate at 3 times the saturation level determined with IMP as substrate.

LIEBERMAN[4] has shown that formation of adenylosuccinate involves bond-formation between the γ-phosphorus of GTP and the oxygen at C-6 of IMP. Evidence has been adduced by MILLER AND BUCHANAN[8] that this reaction might occur in cooperation with attack of the aspartate nitrogen on C-6 of IMP. The present results show that the phosphoester portion of IMP is not only important for specific binding to the IMP site but in addition exerts a profound influence on the maximum velocity of the enzymatic reaction. It is clear that the steric and electronic factors of the phosphoester which modulate specific binding are not identical with those which affect v_{max}. Thus, 5′-thio-IMP, for example, binds as strongly as IMP yet is 15-fold less effective as an acceptor. Previous studies have indicated that binding of IMP is accompanied by a conformational change in the synthetase[1]. It is conceivable that the present IMP analogs reduce v_{max} by steric interference with attainment of the optimum enzyme conformation for rapid C–N and P–O bond formation and/or for rapid product release. It is hence of interest to note that in aqueous solution the preferred conformation of IMP is one in which the phosphate moiety is close to C-8 (refs. 9, 10) and that inspection of a space-filling model shows that in one such conformation the phosphate oxygens can be located as close as two bond distances from the reaction center at C-6 of IMP.

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