Human Hypoxanthine-Guanine Phosphoribosyltransferase

IMP-GMP EXCHANGE: STOICHIOMETRY AND STEADY STATE KINETICS OF THE REACTION*

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Steady state kinetics of hypoxanthine guanine phosphoribosyltransferase-catalyzed reactions are studied. The results obtained suggest that IMP, GMP, P-Rib-PP, and pyrophosphate bind to the same enzyme form, while hypoxanthine and guanine bind to a different form. Guanine activates IMP-pyrophosphorylation. During the reaction guanine and IMP are consumed with formation of GMP and hypoxanthine (IMP-GMP exchange).

The mechanism of purine overproduction "de novo" in the Lesch-Nyhan syndrome and in adult gouty subjects with deficiency of hypoxanthine guanine phosphoribosyltransferase (EC 2.4.9.8, IMP-GMP-pyrophosphate phosphoribosyltransferase) is generally assumed to involve reduced consumption of P-Rib-PP in the phosphoribosyltransferase reaction and a resultant surplus of P-Rib-PP for synthesis of β-phosphoribosylamine and purine de novo (1, 2). However, deficiency of adenine phosphoribosyltransferase (EC 2.4.2.7, AMP:pyrophosphate phosphoribosyltransferase), the closely related enzyme which also operates in the "salvage pathway," is not associated with purine overproduction de novo nor with elevated cellular concentration of P-Rib-PP (3), although the Michaelis constant for P-Rib-PP reported for this enzyme is considerably lower than that for hypoxanthine guanine phosphoribosyltransferase (4). Furthermore a high quantity of P-Rib-PP should normally be utilized by the adenine phosphoribosyltransferase-catalyzed reaction since, in subjects homozygous for deficiency of this enzyme, adenine, 8-hydroxyadenine, and 2,8-dihydroxyadenine account for up to 25% of the total urinary purine metabolites, while adenine and its oxidative products are normally excreted in the urine at levels below 1% of the total urinary purine metabolites (5). Thus the role of hypoxanthine guanine phosphoribosyltransferase in purine metabolism is not yet completely understood.

Recently the steady state kinetics of the forward and reverse hypoxanthine guanine phosphoribosyltransferase-catalyzed reactions have been studied (6). In the present paper, evidence is presented that hypoxanthine guanine phosphoribosyltransferase may have a role in nucleotide interconversion. An IMP-GMP exchange catalyzed by the enzyme is described and the kinetics of the reaction are studied.

EXPERIMENTAL PROCEDURES

Materials

Hypoxanthine guanine phosphoribosyltransferase was purified from human erythrocytes to apparent electrophoretic homogeneity as previously described (6). P-Rib-PP, employed in the final step of the purification procedure to stabilize the enzyme during heat treatment, was removed by exhaustive dialysis against 5 × 10⁻³ M Tris-HCl, 10⁻² M 2-mercaptoethanol, pH 7.4.

All other enzymes, IMP, GMP, hypoxanthine, and guanine were purchased from Boehringer AG.

P-Rib-PP tetrasodium salt was obtained from Sigma Chemical Co. One milligram of the commercial sample contained 1.7 µmol of P-Rib-PP as determined using the enzymatic assay of Kornberg et al. (7). Paper chromatography according to the method of Wood (8) showed no significant impurity in the commercial sample of P-Rib-PP after staining with ammonium molybdate (8).

All other reagents were high purity commercial samples from Merck AG, Fluka AG, and Boehringer AG.

Methods

Hypoxanthine Guanine Phosphoribosyltransferase Assay—IMP pyrophosphorylation (6, 9, 10) was assayed following hypoxanthine production by the continuous spectrophotometric determination at 293 nm of the uric acid formed in the presence of an excess of xanthine oxidase activity (0.08 IU/ml at 37°C) (6). The coefficient of variation of the assay was 5% when 15 measurements were carried out under the same experimental conditions within 2 h.

The steady state level of hypoxanthine in the coupled assay was calculated according to the method of McChure (11). The Kₘ value of xanthine oxidase for hypoxanthine in 0.1 M Tris-HCl buffer, pH 7.4, 0.012 M MgCl₂ at 37°C was found to be 5.5 ± 0.1 S.D. µM. Kₘ and Vₘₚₓ for xanthine oxidase were not appreciably affected by P-Rib-PP, guanine, PPₐ, IMP, and GMP at the concentrations employed in the kinetic experiments.

The synthesis of IMP and GMP from P-Rib-PP and the corresponding purine base was followed spectrophotometrically at 245 and 257.5 nm, respectively, according to the method of Hill (12). In the presence of guanine, IMP synthesis was followed at 240.5 nm. At this wavelength (Fig. 3B) no optical density variation is observed during the conversion of guanine to GMP.

The coefficients of variation were 6.3% for the assay carried out at 257.5 nm, 6.5% for the assay carried out at 245 nm, and 7.5% for the assay carried out at 240.5 nm, when 15 measurements for each assay were performed within 2 h. Under the experimental conditions employed the assays were at least as accurate as the radiochemical assays used by Henderson et al. (4).

The change in extinction for the formation of GMP from guanine in 0.1 M Tris-HCl buffer, pH 7.4, at 37°C was found to be 4570 ± 80 S. D. M⁻¹ cm⁻¹ at 257.5 nm. A value of 2050 ± 50 S. D. M⁻¹ cm⁻¹ at 245 nm was assumed for the change in extinction for the formation of IMP from hypoxanthine (6).

All spectrophotometric measurements were carried out in 0.1 M Tris-HCl, pH 7.4, at 37°C.

The kinetic experiments were performed using a Perkin-Elmer two-wavelength spectrophotometer model 356. The noise was less than 0.0005 A and the total system drift was less than 0.001 A/h.

Free Mg²⁺ concentration, computed as previously described (6), was held practically constant (0.012 M). A pKₘ value for GMP of 6.66 (13) and a stability constant for MgGMP complex of 1.1 × 10¹⁵ M⁻¹ (14, 15) were assumed. The concentration of free Mg²⁺ in the presence of P-Rib-PP was calculated without taking into account the binding of the metal ion with P-Rib-PP since in all experiments the total concentration of this substrate was kept at least 20 times lower than [Mg₂⁺].
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GMP Assay—GMP concentration in the hypoxanthine guanine phosphoribosyltransferase-reaction mixture was measured by the NADH coupled enzyme method of Grasal (16), after protein precipitation according to Hurlbert et al. (17). One-milliliter of the sample was added to 1 ml of 1 m ice-cold perchloric acid. The precipitated protein was removed by centrifugation. A 1.5 volume of the supernatant was neutralized by adding 0.3 ml of a solution containing 0.5 m triethanolamine-HCl and 2 m K2CO3. After incubation in an ice bath for 10 min, the potassium perchlorate precipitate was removed by centrifugation and the supernatant was used for the quantitative determination of GMP. The reaction mixture contained 1 ml of the deproteinized sample, 1 pmol of ATP, 1 pmol of phosphoenolpyruvate, 0.25 pmol of NADH, 0.12 pmol of MgСO4, 130 pmol of KCl, 3 IU of lactate dehydrogenase, 3 IU of pyruvate kinase in a final volume of 1.27 ml. A first spectrophotometric reading was made at 340 nm to determine the initial absorbance. A 20-μl volume of 20 IU/ml of guanylate kinase was then added to the mixture and the reaction was followed to completion at 340 nm. GMP concentration was calculated from the optical density variation at 340 nm assuming a molar extinction coefficient of 6220 for NADH and considering that 2 mol of NAD are formed from 1 mol of GMP (16).

**Purine Base Assay**—Hypoxanthine guanine phosphoribosyltransferase-catalyzed reaction was stopped by addition of 0.2 ml of 0.25 m EDTA to 1 ml of the reaction mixture (4). Hypoxanthine and guanine concentrations in the stopped reaction mixture were measured by the enzymic spectrophotometric methods of Kalkkar (18).

**RESULTS AND DISCUSSION**

**Hypoxanthine Guanine Phosphoribosyltransferase-catalyzed IMP-GMP Exchange**—When hypoxanthine guanine phosphoribosyltransferase-catalyzed IMP pyrophosphorolysis was followed spectrophotometrically at 293 nm in the presence of an excess of xanthine oxidase activity (0.08 IU/ml) (6), an increase of the initial reaction rate was observed in the presence of guanine. The maximal value of the initial reaction rate was reached at very low guanine concentrations. Increasing guanine concentration above 1.5 × 10-6 m gave no further enhancement in the initial velocity. After a period of time almost linearly related to the initial guanine concentration, the slope of the progress reaction curve decreased sharply to the value observed in the absence of guanine (Fig. 1). The optical density variation corresponding to the initial burst was a linear function of guanine concentration (Fig. 1, inset). IMP, PPi, magnesium ions, hypoxanthine guanine phosphoribosyltransferase, and xanthine oxidase were necessary for the reaction to occur. A decrease in optical density at 293 nm up to around the initial absorbance values was observed upon addition, at the end of the reaction, of 10 μl of 18 IU/ml of uricase to 1 ml of the reaction mixture suggesting that uric acid was the chief end product absorbing at 293 nm (18).

In order to study if the sharp bending of the reaction progress curve in the presence of guanine could be attributed to the consumption of this compound, the following experiment was carried out. Twenty milliliters of a solution containing 0.1 m Tris-HCl, pH 7.4, 0.012 m MgСl2, 1 × 10-5 m guanine, 5 × 10-5 m IMP, 5 × 10-4 m PPi, 0.08 IU/ml of xanthine oxidase, and 0.5 ml of a standard hypoxanthine guanine phosphoribosyltransferase preparation were incubated at 37°C. Guanine concentration was determined on 1-ml aliquots taken from the reaction mixture at different periods of time as described under “Experimental Procedures.” As Fig. 2A shows, guanine was consumed during the reaction. The rate of consumption of the purine base was constant over the interval studied.

**Hypoxanthine guanine phosphoribosyltransferase** in the presence of P-Rib-PP and guanine catalyzes the formation of GMP and PPi (4). P-Rib-PP is formed during IMP pyrophosphorolysis (6). Therefore the possibility that guanine consumption during IMP pyrophosphorolysis could be attributed to the formation of GMP was explored. Samples of 1 ml of the reaction mixture employed to demonstrate guanine consumption were removed at various periods of time and GMP determination was performed as described above. As Fig. 2A shows, GMP was formed during the reaction. The rate of formation of the nucleotide was constant over the interval studied and, at any time, GMP concentration was within the experimental error, equal to the decrease in guanine concentration. Under the experimental conditions employed above, the rate of GMP formation and guanine consumption was proportional to hypoxanthine guanine phosphoribosyltransferase concentration in the range studied (from 0.025 to 0.2 ml of a standard enzyme preparation in 1 ml of the reaction mixture).

Using the same buffer, magnesium ion and substrate concentrations and the incubation temperature described above, in the presence of guanine, IMP pyrophosphorolysis associated with GMP formation also occurs in the absence of xanthine oxidase. Under these conditions, as Fig. 2B shows, guanine was consumed with formation of an equimolar amount of GMP and hypoxanthine. The latter base, substrate of xanthine oxidase, was identified as hypoxanthine by thin layer chromatography (19) and by its absorbance spectra (20).

**Fig. 1.** Progress reaction curve of IMP pyrophosphorolysis in the absence (b) and in the presence of 5.5 × 10-4 m guanine (a). The concentrations of IMP and PPi were 1 × 10-4 m and 5 × 10-4 m, respectively. All other conditions were as described under “Experimental Procedures.” Inset, optical density variation corresponding to the initial burst as a function of guanine concentration.

**Fig. 2.** Variation of the concentration of guanine (A), hypoxanthine (B), and GMP (C) during IMP pyrophosphorolysis in the presence of 1 × 10-4 m guanine. The reaction was carried out in the absence (A) and in the presence of 0.08 IU/ml of xanthine oxidase (A). The experimental conditions were as described in the text.
after purification on Dowex 1 according to the method of Cohn (21).

The stoichiometry of the reaction was substantiated by the following spectrophotometric measurements. Fig. 3A shows differential absorption spectra recorded at 20-min intervals between the solution employed to demonstrate IMP pyrophosphorylase activity and the reference cell. These results are in good agreement with those obtained using hypoxanthine as substrate (6) and suggest an obligatory order of substrate binding, first P-Rib-PP and then guanine, with products dissociating from the enzyme in a random order.

Using GMP as alternate substrate of the hypoxanthine guanine phosphoribosyltransferase-catalyzed reaction between guanine and P-Rib-PP, to form GMP and PPi, in the presence of magnesium ions has been recently studied (6). The minimum kinetic model which still fits all the experimental data is that MgIMP and one or both monomagnesium complexes of PPi bind to the enzyme in a rapid equilibrium random fashion while products dissociate from enzyme in an ordered sequence, first hypoxanthine and then the magnesium complex(es) of P-Rib-PP.

The hypoxanthine guanine phosphoribosyltransferase-catalyzed reaction between guanine and P-Rib-PP to form GMP and PPi, in the presence of magnesium ions has been studied. According to Henderson et al. (4) and to our results GMP inhibition was competitive with respect to P-Rib-PP while GMP and PPi, inhibitions were noncompetitive with respect to guanine. As shown in Fig. 4, PPi was competitive with respect to P-Rib-PP. The inhibition patterns suggest that GMP, pyrophosphate, and P-Rib-PP bind to the same enzyme form, while guanine binds to a different one. These results are in good agreement with those obtained using hypoxanthine as substrate (6) and suggest an obligatory order of substrate binding, first P-Rib-PP and then guanine, with products dissociating from the enzyme in a random order.

Using GMP as alternate substrate of the hypoxanthine guanine phosphoribosyltransferase-catalyzed IMP pyrophosphorylase, it was found that GMP inhibition was competitive with respect to IMP (Fig. 5). For the reverse reaction, guanine inhibition was competitive with respect to hypoxanthine in good agreement with the results of Henderson et al. (4).

Therefore nucleotides must bind to the same enzyme form while purine bases to different ones.

Considering the overall reaction in the presence of both nucleotides and purine bases, the minimum kinetic model which still fits the experimental data reported above is shown in Fig. 6 (solid lines). For the hypoxanthine guanine phosphoribosyltransferase-catalyzed IMP pyrophosphorylase it has been assumed that the binding and dissociation rate constants of both nucleotide and pyrophosphate for the free enzyme are much faster than the subsequent reactions (6). Since GMP inhibition against IMP is linear (Fig. 5, slope replot), it has been assumed that also guanylate adds to the free enzyme in a rapid equilibrium fashion. The complete steady state rate equation for this mechanism was derived by the procedure of Henderson et al. (4) obtained a noncompetitive inhibition by pyrophosphate with respect to P-Rib-PP at low, fixed guanine concentration with straight lines strongly converging near the ordinate axis. The discrepancy between this experimental finding and ours could be attributed, at least in part, to the different concentration of magnesium employed. In Ref. 4, all assays were performed at a constant concentration of MgSO4 (10^{-3} M) while P-Rib-PP ranging from 3.3 x 10^{-4} to 2 x 10^{-4} M was always added as the dimagnesium salt. Under these conditions the total concentration of magnesium was varying and therefore the concentration of magnesium complex(es) of P-Rib-PP which is (are) substrate(s) of the enzyme could be not proportional to the total amount of P-Rib-PP present in solution (6). Considering the initial velocity pattern in the absence of noncompetitive inhibition by PPi with respect to P-Rib-PP obtained by Henderson et al. (4) cannot be taken as evidence supporting an ordered mechanism for the reaction as previously discussed (6).

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**Fig. 3.** Differential absorption spectra recorded at 20-min intervals of the hypoxanthine guanine phosphoribosyltransferase-catalyzed reactions. A, IMP + guanine + hypoxanthine + GMP. The sample cell contained the following reaction mixture: 0.1 M Tris-HCl, pH 7.4, 0.012 M MgCl2, 1 x 10^{-4} M guanine, 5 x 10^{-5} M IMP, 5 x 10^{-4} M PPi, and 0.02 ml of a hypoxanthine guanine phosphoribosyltransferase preparation in a final volume of 2 ml. The reference cell contained the same compounds without PPi. The theoretical differential spectrum (dotted line) was calculated as described in the text. B, guanine + P-Rib-PP + GMP + PPi. The sample cell contained the following reaction mixture: 0.1 M Tris-HCl, pH 7.4, 0.012 M MgCl2, 1 x 10^{-4} M guanine, 1 x 10^{-5} M P-Rib-PP, and 0.02 ml of a hypoxanthine guanine phosphoribosyltransferase preparation in a final volume of 2 ml. The reference cell contained the same compounds without P-Rib-PP. C, hypoxanthine + P-Rib-PP → IMP + PPi. The sample cell contained the following reaction mixture: 0.1 M Tris-HCl, pH 7.4, 0.012 M MgCl2, 1.7 x 10^{-4} M hypoxanthine, 1 x 10^{-3} M P-Rib-PP, and 0.02 ml of a hypoxanthine guanine phosphoribosyltransferase preparation in a final volume of 2 ml. The reference cell contained the same compounds without P-Rib-PP.
The reaction rate of hypoxanthine guanine phosphoribosyltransferase-catalyzed IMP pyrophosphorolysis increases (Fig. 1). According to Equation 1 this occurs when Guanine inhibition is noncompetitive with respect to P-Rib-PP against PP; at a fixed concentration of IMP is noncompetitive with respect to M; at 1 \times 10^{-3} M; \bullet 2 \times 10^{-3} M. All other conditions were as described under "Experimental Procedures." Inset, slope 1/IMP replot with respect to molar concentration of GMP.

Fig. 5 (center). Alternate substrate inhibition of the reaction IMP + PP\_i \rightarrow hypoxanthine + P-Rib-PP by GMP with IMP as the variable substrate. The concentration of PP\_i was held constant at \times 10^{-3} M. The concentration of GMP were: ■ none; □, 4 \times 10^{-5} M; △, 1.2 \times 10^{-4} M; ○, 2.3 \times 10^{-4} M; ●, 4.6 \times 10^{-4} M. All other conditions were as described under "Experimental Procedures." Inset, slope 1/IMP replot with respect to molar concentration of GMP.

In accord with the prediction of Equation 1, the ratio, k_{12}/k_{10} + k_{18}, of the maximal increase in the initial rate of IMP pyrophosphorolysis when hypoxanthine and P-Rib-PP are used as substrates, GMP inhibition is competitive with respect to IMP and P-Rib-PP, is equal to 1/\gamma, the ratio of the steady state level of hypoxanthine to the initial substrate concentration (Fig. 1).

In the absence of IMP and PP\_i, at a constant concentration of the second substrate and at different fixed concentrations of guanine, should give, according to Equation 1, families of parallel lines. However, as shown in Fig. 9, lines converging far to the left of the 1/\gamma axis have been obtained.

The maximal increase in the initial rate of IMP pyrophosphorolysis in the presence of guanine was achieved at concentrations of the purine base lower than 1.5 \times 10^{-6} M. Since guanine activation cannot be followed spectrophotometrically at very low concentrations of this compound, owing to its consumption during the reaction, the experiments shown in Fig. 9 have been carried out only in the absence and at saturating concentrations of guanine. The same result has been obtained using different saturating concentrations of guanine (ranging from 1.5 \times 10^{-6} M to 1 \times 10^{-4} M).

The steady state level of hypoxanthine (<4 \times 10^{-6} M) in the xanthine oxidase-coupled assay for IMP pyrophosphorolysis appears negligible with respect to the value that can justify the deviation from parallel of the lines obtained in Fig. 9.

According to Equation 1, the ratio, \gamma, between the slope of the reciprocal plot of the initial velocity against the concentration of IMP or PP\_i, at a constant concentration of the other substrate, should give, according to Equation 1, families of parallel lines. However, as shown in Fig. 9, lines converging far to the left of the 1/\gamma axis have been obtained.

The maximal increase in the initial rate of IMP pyrophosphorolysis in the presence of guanine was achieved at concentrations of the purine base lower than 1.5 \times 10^{-6} M. Since guanine activation cannot be followed spectrophotometrically at very low concentrations of this compound, owing to its consumption during the reaction, the experiments shown in Fig. 9 have been carried out only in the absence and at saturating concentrations of guanine. The same result has been obtained using different saturating concentrations of guanine (ranging from 1.5 \times 10^{-6} M to 1 \times 10^{-4} M).

In the absence of GMP, hypoxanthine, and P-Rib-PP, the concentration of PP\_i was held constant at 1 \times 10^{-3} M. The concentration of IMP or PP\_i was: ■ none; □, 4 \times 10^{-5} M; △, 1.2 \times 10^{-4} M; ○, 2.3 \times 10^{-4} M; ●, 4.6 \times 10^{-4} M. All other conditions were as described under "Experimental Procedures." Inset, slope 1/IMP replot with respect to molar concentration of GMP.

Fromm (22) and Cha (23) and is given in Equation 1 using Cleland’s nomenclature.²

In accord with the prediction of Equation 1, GMP inhibition is competitive with respect to P-Rib-PP against PP; at a fixed concentration of IMP is noncompetitive with respect to M; at 1 \times 10^{-3} M; \bullet 2 \times 10^{-3} M. All other conditions were as described under "Experimental Procedures." Inset, slope 1/IMP replot with respect to molar concentration of GMP.

![Diagram](http://www.jbc.org/content/10235/S5)
This result can be taken as experimental evidence that, under the conditions employed, the steady state concentration of the purine base does not appreciably affect the initial rate of IMP pyrophosphorolysis.

The slight deviation from parallel of the lines obtained in Fig. 9 cannot be therefore attributed to the presence of hypoxanthine and supports that the real mechanism may be some what more complicated than that reported in the King-Altman scheme.

According to the general Cleland's rules (24) for predicting initial velocity patterns, guanine affects the slope of reciprocal plots shown in Fig. 9 if it and the varied substrate combine reversibly with the same enzyme form or with different forms that are separated in the reaction sequence by freely reversible steps. As previously discussed, guanine binds to a different form than IMP and PP. According to the reaction scheme reported above, in the absence of P-Rib-PP, GMP, and of significant amounts of hypoxanthine, the points of addition of IMP and PP, are separated from the point of addition of guanine by the irreversible step of hypoxanthine release. Although more complex mechanisms cannot be excluded, the simplest assumption to eliminate the irreversible step which separates the points of addition of the substrates is that guanine can bind to the enzyme also before the release of hypoxanthine. Taking into account that guanine and hypoxanthine bind to the same enzyme form, a Theorell-Chance mechanism (25) for guanine binding and hypoxanthine release could be postulated (Fig. 6).

REFERENCES
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