THE RATE-LIMITING STEP IN THE ADENINE PHOSPHORIBOSYLTRANSFERASE REACTION*

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Summary. Measurement of the effect of $^{2}H_2O$ on the maximum velocity of adenine phosphoribosyltransferase suggests that removal of the proton from the 9-position of adenine is rate-limiting for this reaction. Studies of the effects of pH and of temperature on maximum velocity suggest that removal of this proton occurs by general base catalysis involving protein-bound histidine.

Adenine phosphoribosyltransferase (adenylate: pyrophosphate phosphoribosyltransferase, ED 2.4.2.7) catalyzes an ordered reaction in which a ternary enzyme:Mg-PP-ribose-P:adenine complex is converted into an enzyme:Mg PP_i:AMP complex (1). In the catalytic process, the bond between the 1-carbon of PP-ribose-P and the oxygen attached to it is broken, the proton on the 9-nitrogen of adenine is removed, and the bond between the 9-nitrogen of adenine and the 1-carbon of ribose-5-P is formed. A study has been made to determine which step is rate limiting, and what group(s) on the enzyme might participate in the catalysis.

Methods. The initial velocity of radioactive AMP synthesis from Mg-PP-ribose-P and adenine-^{14}C was measured as described elsewhere (2) with an enzyme preparation partially purified from Ehrlich ascites tumor cells (3).

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In order to measure the rate of the adenine phosphoribosyltransferase reaction in ^2H_2O, $MgSO_4$ and Tris-HCl were dissolved in this solvent (New England Nuclear Corp.). The apparent p^2H of the buffer was measured with a pH meter, and as suggested by several groups (4,5,6), 0.4 unit was then added to this figure to obtain the true p^2H value. Substrates were prepared by diluting concentrated H_2O solutions of PP-ribose-P and adenine-^{14}C in ^2H_2O. The final reaction mixtures contained more than 99% ^2H_2O.

Results and discussion. During the catalytic process the proton must be removed from the 9-nitrogen of adenine and a glycosidic bond must be formed. The possibilities were considered (a) that removal of the proton was the rate-determining step, or (b) that the nucleophilic attack by the 9-nitrogen on the 1-carbon of PP-ribose-P was the rate-determining step. Comparison of reaction rates in H_2O and ^2H_2O was used to distinguish between these two possibilities. Removal of the proton by a general base is the rate-determining step in general base catalysis. A nucleophilic attack may occur subsequent to this proton removal, but it must be faster than the attack by the base. For those cases in which the rate-limiting step is the attack by the nucleophile and subsequent break-down of the intermediate, a proton may be lost or gained, but its transfer is faster than the nucleophilic attack.

Maximum velocities and Michaelis constants were determined from secondary plots (Figure 1) obtained from double reciprocal plots of initial velocity versus substrate concentration at pH 8.1 in H_2O and ^2H_2O. (Bender et al. (7) observed that the pK_a's of protein functional groups are 0.5 $\pm$ 0.2 pH units higher in ^2H_2O than in H_2O, and the pH was therefore chosen to be 2

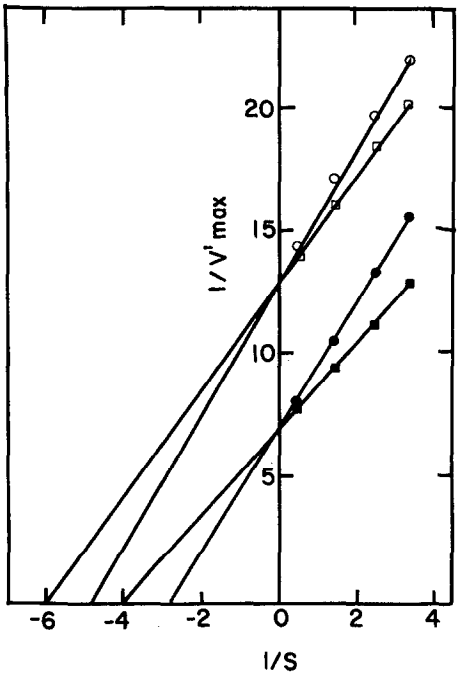

Figure 1. Effect of ^2H_2O on maximum velocity and Michaelis constants. Initial velocity was measured as a function of PP-ribose-P concentration and of adenine concentration at a series of fixed concentrations of the other substrate in 0.1 M Tris buffer, pH 8.1, and 10^{-3} M $MgSO_4$, in H_2O and ^2H_2O. Ordinates of double reciprocal plots of these data are replotted against substrate concentration. PP-ribose-P is variable substrate in H_2O (■) and ^2H_2O (□); adenine is variable substrate in H_2O (●) and ^2H_2O (○).

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units above the group on the enzyme whose ionization affects the maximum forward velocity, V_1, in order to avoid this complication.)

The ratio of $V_1(H_2O)/V_1(^2H_2O)$* was found to be 1.84, $K_A(H_2O)/K_A(H_2O)$ was 1.55, and $K_B(H_2O)/K_B(^2H_2O)$ was 1.70. Isotope effects on K_A and K_B were expected because both Michaelis constants contain V_1 terms. Wiberg (8) has, on theoretical grounds, postulated that general base catalysis

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* The abbreviations used are V_1, maximum forward velocity; K_A, Michaelis constant for PP-ribose-P; K_B, Michaelis constant for adenine; S, substrate.

should proceed faster in H_2O than in ^2H_2O. Bender, Pollik and Neveau (9) observed that general-base-catalyzed reactions in which the base was imidazole gave an isotope effect greater than two, whereas in nucleophilic reactions catalyzed by imidazole no isotope effect was observed. The isotope effect on maximum velocity observed in these experiments is believed to be of sufficient magnitude to be consistent with a mechanism involving removal of the proton from the 9-position of adenine as the rate-limiting step.

Maximum velocities at 10°, and 30° and at various pH values between 5.5 and 11 were obtained from double reciprocal plots of initial velocity versus substrate concentration. These data are shown in Figure 2. At 30° the apparent ionization constant was 6.05, and at 10° it was 6.25. Because PP-ribose-P has an ioni-

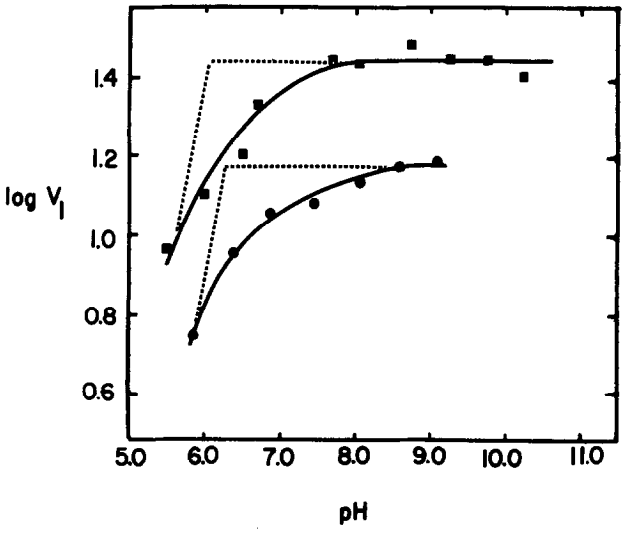

Figure 2. Effect of pH and temperature on logarithm of maximum velocity. Initial velocity was measured as a function of PP-ribose-P concentration and of adenine concentration at a series of fixed concentrations of the other substrate in 0.15 M histidine-Tris-glycine buffer and 10^{-3} M $MgSO_4$ at 10° (●), and 30° (■). Different enzyme concentrations were used at each temperature. Maximum velocity was obtained from replots of ordinates of double reciprocal plots of these data. Dotted lines represent extrapolations of straight portions of these curves.

zation constant much lower than these values (pK_a = 4.7), and adenine has pK_a's of 4.2 and 9.8, the ionization constants determined in these experiments are believed to be due to the ionization of an amino acid residue on the enzyme. The pK_a values obtained are close to those found in other proteins at 25° for the imidazole group in histidine, 5.6 to 7.0, but slightly lower than those of N-terminal α-amino residues, 6.5 to 8.5, and higher than those of carboxyl groups, 3.0 to 4.7 (10).

The apparent heat of ionization calculated from the change in ionization constant between 10° and 30° according to the equation, $\Delta H = -2.303RT^2 (dpK_a/dT)$, was +6,140 cal $mole^{-1}$. This

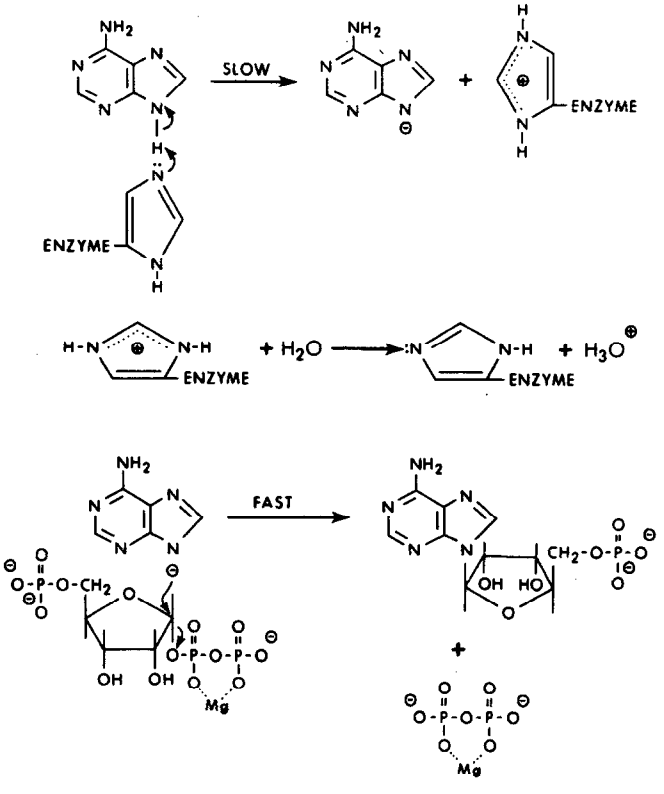

Figure 3. Proposed mechanism of the adenine phosphoribosyltransferase reaction.

value is close to the +6,900 to +7,500 cal $mole^{-1}$ reported for the imidazole group of histidine, and significantly different from those for α-amino groups, +10,000 to -13,000 cal $mole^{-1}$, and those for carboxyl groups, -1,500 cal $mole^{-1}$ to +1,500 cal $mole^{-1}$ (10). Both the pK and ΔH data are consistent with the involvement of an enzyme-bound histidine in the catalytic process.

Removal of the proton on the 9-position of adenine by a general base catalyzed reaction involving histidine should facilitate attack by the 9-nitrogen on the anomeric carbon of PP-ribose-P, the latter having been made strongly electrophilic by the pyrophosphoryl group. Anionic-anionic repulsion between the now charged adenine and the pyrophosphate should also aid displacement of the latter from the enzyme. The basic features of the proposed mechanism are shown in Figure 3.

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