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The pH Dependence of Rat liver RNA Polymerases I and II

Dependencia al pH de las RNA polimerasas I y II de hígado de rata

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The effect of pH on the stability and activity of rat liver RNA polymerases I (A) and II (B) has been studied. Both enzymes are irreversibly inactivated in buffer solutions below pH 5.0. K_m values of the two enzymes are constant between pH 6.5 and 8.7 but a two —to three— fold increase is observed between pH 8.7 and 9.7. The V_{max} versus pH profiles are bell-shaped curves indicating the participation of two ionizing groups with apparent pK_a values of 6.5 and 9.8 for enzyme I and 6.7 and 9.9 for enzyme II. Both enzymes are inactivated by photooxidation in the presence of Rose Bengal. It is suggested that the above pK_a corresponds to the imidazole of a histidine residue and an amino group of a lysine residue.

The knowledge of the amino acid residues involved in the catalytic process is a fundamental requisite for the construction of a reaction mechanism involving the enzyme molecule. Two main routes are available to obtain such information. One is the identification by means of a chemical modification which can be correlated with a change in activity. Another is the study of the reaction under conditions which affect the ionization of one or more of the participating amino acid residues. From the latter studies, the pK_a values for the ionizable groups may be obtained and the corresponding amino acid residues which participate in the catalytic steps may thus be identified. Both efforts are being undertaken in our laboratory in the study of eucaryotic DNA-dependent RNA polymerases.

Eucaryotic cells have multiple nuclear RNA polymerases which can be distinguished by their chromatographic behaviour in DEAE-Sephadex (1) or by their sensitivity to the mushroom toxin alpha-amanitin (2,3). The various purified enzymes have been characterized by their template specificity, preferential divalent metal ion activation, ionic strength and subunit composition (4). Despite the wealth of this information, little is known concerning the mechanism of catalysis.

We have previously reported that *Escherichia coli* (5) and rat liver RNA polymerases I and II (6) are rapidly inactivated by pyridoxal 5'-phosphate. The inhibition is reversed by amines and can be made irreversible by reduction with sodium borohydride. Spectral and chemical data indicated that the inactivation is due to the formation of a Schiff base between the inhibitor and ε-amino groups of lysine residues (5, 6). The reactive subunits have been identified in yeast RNA polymerase I (7).

This report is concerned with the effect of pH on the stability and activity of rat liver

RNA polymerses I and II. We have found that both enzymes are irreversibly inactivated when incubated below pH 5. Both enzymes have very similar K_m versus pH and V_{max} versus pH profiles. The V_{max} versus pH profiles are bell-shaped curves, indicating the participation of two ionizing groups with apparent pK_a values of 6.5 and 9.8 for enzyme I and 6.7 and 9.9 for enzyme II. Both enzymes are inactivated by photo-oxidation in the presence of Rose Bengal at pH 7.9. It is suggested that the above pK_a could correspond to the imidazole of a histidine residue and an amino group of a lysine residue.

MATERIALS AND METHODS

Chemicals. ^{3}H-UTP (20 Ci/nmole) was obtained from Amersham/Searle Corp., Illinois. Nucleoside triphosphates, dithiothreitol and calf thymus DNA were from Sigma Chemical Co., St. Louis, Mo. Rose Bengal was a product from Aldrich Chemical Co., Milwaukee, Wisconsin. Alpha-amanitin was from Boehringer, Germany. Glycerol, buffer salts and ammonium sulfate were analytical grade reagents.

Enzyme Purification. Rat liver RNA polymerases I and II were purified from isolated nuclei by a procedure described elsewhere (6). Enzyme I has a specific activity of 30 units/mg of protein and enzyme II, 60 units/mg (one unit of activity is defined as 1/nmole of UMP incorported in 10 min at 30°).

Enzyme Assay. RNA polymerases were assayed in a final volume of 0.06 ml. The standard reaction mixture contained 50 mM Tris-HCl pH 7.9, 12.5% glycerol, 2.5mM $MgCl_2$, 1.6 mM $MnCl_2$, 6 mM NaF, 0.6 mM each ATP, GTP and CTP, 0.02 mM UTP, 0.4 μCi ^{3}H-UTP, 0.3 mM dithiothreitol, 0.05 mM EDTA and 12 μg of native calf thymus DNA. Enzyme I was assayed at 0.05 M $(NH_4)_2SO_4$ and enzyme II at 0.12 M $(NH_4)_2SO_4$. After incubation for 10 min at 30°, 0.05 ml of the reaction mixture was analyzed for ^{3}H-RNA using DE-81 filters as described previously (6).

RESULTS AND DISCUSSION

Effect of pH on the Stability of Rat Liver RNA Polymerases

Before studying the effect of pH on the activity of the enzymes, it was of interest to examine the stability of polymerases at different pH values. For that reason, RNA polymerases I and II were incubated at 0° in several buffers of desired pH. At appropriate time intervals, aliquots were withdrawn and assayed at pH 7.9. The results obtained are shown in Figure 1. Upon incubation of the enzymes below pH 6, a time-dependent inactivation takes place. The rate of inactivation increases sharply as the pH decreases. Both enzymes are affected in a similar way, although polymerase I seems to be slightly more susceptible. No reactivation was obtained after short or prolonged incubation of the partially inactivated enzymes at neutral pH. A similar phenomenon has been observed in our laboratory with homogeneous RNA polymerase I from yeast (Bull, Campino, Bell,

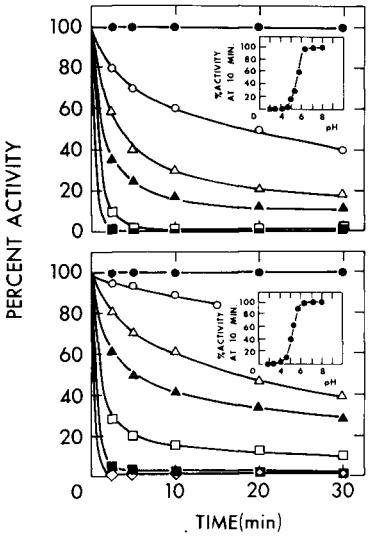

Fig. 1. Irreversible inactivation of rat liver RNA polymerases I and II at acid pH. Enzyme (4 μg) was incubated at 0° in buffers of desired pH (0.01 M formate, acetate, Tris-maleic and Tris-HCl); at different time intervals aliquots were withdrawn and assayed at pH 7.9 as described under Materials and Methods. Upper figure corresponds to enzyme I; lover figure to enzyme II. ●---●: pH 7.0, ○---○: pH 6.0, △---△: pH 5.4, ▲---▲: pH 5.0, □---□: pH 4.5, ■---■: pH 4.0. The inset shows the percent activity at 10 min incubation. 100% corresponds to 10 nmoles UMP incorporated per ml per 10 min.

Venegas and Valenzuela, manuscript in preparation).

The observed denaturation of the enzymes at acid pH could be due to the loss of the complex quaternary structure of the enzymes. The transition occurs between pH 6 and 4 where protonation of carboxylic side chains are likely to occurs. Polymeric proteins tend to dissociate at low pH as a consequence of electrostatic repulsion due to the excess of positive charges. An alternative explanation would be the loss of a divalent metal ion necessary for activity (8). For instance, it has been demonstrated for yeast RNA polymerase I that it is a Zn metalloenzyme (9). At acid pH protons will be able to compete with the metal for the enzyme ligands, as has been found with other metalloenzymes. The irreversible loss of activity of rat liver and other eucaryotic RNA polymerases at acid pH may explain in part the low yield and decreased stability of eucaryotic enzymes when subjected to $(NH_4)_2SO_4$ fractionation without carefully controlling the pH of the solution.

Effect of pH on the Activity of Rat Liver RNA Polymerases

The pH may affect the activity of an enzyme by modifying the K_m of the substrates or by altering the V_{max} of the reaction. The effect on the K_m was examined by performing the reaction at different pH values using CTP as the variable substrate while the other nucleotide substrates were held constant. CTP was selected in order to avoid possible kinetic complications if initiating nucleotides (as ATP or GTP) are used, as has been shown with *E. coli* RNA polymerase (10). K_m values, obtained from double reciprocal plots (11), are shown in Table I. For both enzymes, there is no significant variation between pH 6.5 and 8.7 but the two —to three— fold increase observed at higher pH values is similar to that reported by others for rat liver (12) and calf thymus enzymes (13).

The results of the effect of pH on V_{max} are shown in Figure 2 and Figure 3 for polymerases I and II respectively. Both enzymes have a rather similar pH-rate profile:

TABLE I

Effect of pH on the K_m of Rat Liver RNA Polymerases I and II

pH	*Buffer*	$K_m \times 10^5$ *M*
Enzyme I		
6.53	Tris-maleic acid	1.18
7.27	Tris-maleic acid	1.12
7.81	Tris-HCl	0.95
8.39	Tris-HCl	1.02
9.77	Glycine	3.32
Enzyme II		
7.05	Tris-maleic acid	2.02
7.69	Tris-HCl	1.64
7.97	Tris-HCl	1.50
8.30	Tris-HCl	1.42
8.50	Tris-HCl	1.45
8.75	Tris-HCl	2.71
9.07	Glycine	3.81
9.31	Glycine	4.21

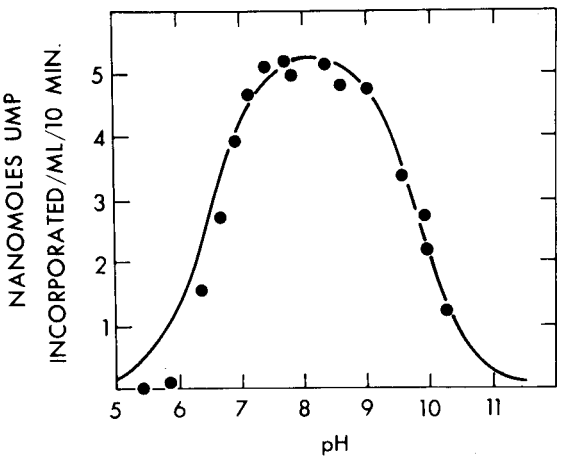

Fig. 2. V_{max}-pH profile for the activity of rat liver RNA polymerase I. Enzyme (4 μg) was assayed in buffers of desired pH (0.05 M Tris-maleic, Tris-HCl and glycine) as described under Materials and Methods. The solid line is a theoretical curve calculated using pK_a values of 6.5 and 9.8 and a V_{max} (lim) of 5.25 nmoles of UMP incorporated per ml in 10 min. The experimental points are the average of 3 determinations which agreed between 5 and 10%.

They are not active below pH 6; between pH 6 and 8 there is a sharp increase in V_{max}, which reaches a maximum around pH 8-8.5, and between 8.5 and 10 the activity decreases again. The best fit to the experimental points

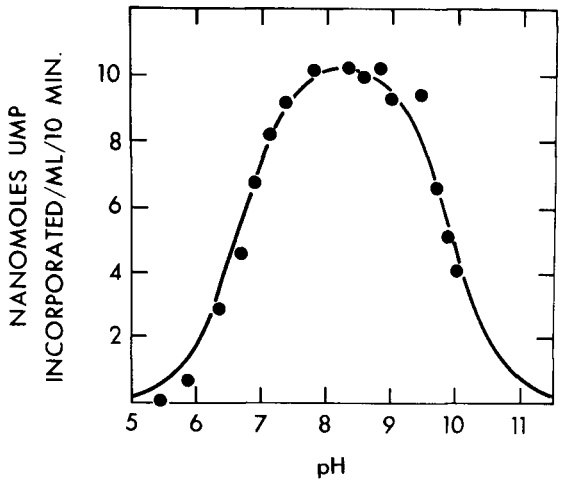

Fig. 3. V_{max}-pH profile for the activity of rat liver RNA polymerase II. Enzyme (4 μg) was assayed in buffers of desired pH (0.05 M Tris-maleic, Tris-HCl and glycine) as described under Materials and Methods. The solid line is a theoretical curve calculated using pK_a values of 6.7 and 9.9 and V_{max} (lim) of 10.5 nmoles of UMP incorporated per ml in 10 min. The experimental points are the average of 3 determinations which agreed between 5 and 10%.

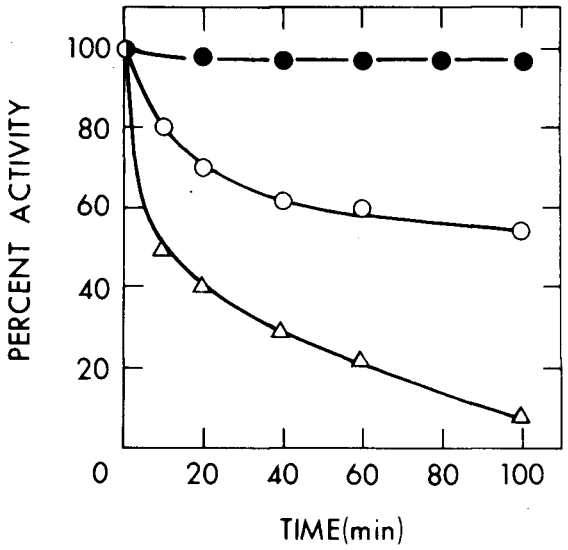

Fig. 4. Inactivation of rat liver RNA polymerases I and II by photooxidation in the presence of Rose Bengal. Enzymes (0.3-0.5 mg/ml) were incubated with 1.62×10^{-6} M Rose Bengal at 0° in 0.05 M Tris-HCl pH 7.9, 25% glycerol and 0.1 M KCl and illuminated with a 150 watt lamp at 40 cm of distance. Aliquots containing 3-5 μg of enzymes were taken at different times and assayed as described under Materials and Methods. 100% of activity corresponds to the incorporation of 240 pmol of UMP into RNA. Enzyme I: o---o; enzyme II: Δ---Δ; enzymes I and II in the dark: •---•.

was obtained using pK_a values of 6.5 and 9.8 for enzyme I and 6.7 and 9.9 for enzyme II. Similar curves have been reported by Gissinger *et al.* (13) for calf thymus RNA polymerases I and II.

Photooxidation in the Presence of Rose Bengal

The acid limbs of the curves suggest the participation of the imidazole group of histidine. This hypothesis is further suggested by photooxidation experiments with Rose Bengal. In the presence of light both enzymes are rapidly inactivated by low concentrations of the dye. Figure 4 shows the kinetics of inactivation of the enzymes by 1.6×10^{-6}M Rose Bengal. It is clear that one enzyme (II) is more susceptible to Rose Bengal inhibition than the other (I) at the same dye concentration. This could be due to a more efficient binding of the modifying agent. In the absence of light the enzymes are not inhibited. Preliminary experiments with homogeneous yeast RNA polymerase I which is also inhibited by photooxidation with Rose Bengal indicate that the DNA binding step is not affect by photooxidation (Campino, Bull, Venegas and Valenzuela, unpublished results). Ishihama and Hurwitz have found that *E. coli* RNA polymerase is inactivated by photooxidation by Rose Bengal and the step affected is the elongation of the RNA chain (14). None of the evidence presented for the involvement of histidine residues in the action of rat liver RNA polymerases would by itself carry much conviction, yet taken together they seem reasonably persuasive and form a basis for the design of substrate— and template-like reagents reactive towards imidazole side chains. Indeed, the imidazole group is very often involved in enzyme action and in RNA polymerases can participate as acid-base or nucleophilic catalyst in the polymerization step by assisting the attack of the terminal 3' OH of the nascent chain to the pyrophosphate bond of the next nucleotide substrate.

The pK_a 9.7 and 9.9 found for enzymes I and II may be associated with the ionization of an ε-amino group of a lysine residue. The participation of this group in the reactions of

rat liver RNA polymerases has been demonstrated by chemical modification with pyridoxal 5'-phosphate (6). It is suggested that a protonated lysine amino group participates in the binding of nucleotide substrate or DNA to the enzyme by ionic interactions with the phosphate groups. The increase of the K_m values above pH 8.7 agrees with this proposal.

More detailed structural and kinetic experiments are necessary to prove these proposals. Studies on the effect of pH on the individual steps (i.e., DNA binding, initiation, elongation and termination) will help to determine the role of the proposed enzyme groups in the catalytic process. Some of these studies are in progress in our laboratory.

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