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Imidazole Catalysis of Amino Proton Exchange in 2′,3′-Cyclic Adenosine Monophosphate. A General Exchange Mechanism†

Bruce McConnell

ABSTRACT: Imidazole and 2-methylimidazole increase the proton magnetic resonance (pmr) line widths of the amino protons of 2′,3′-cAMP. This amine-induced broadening is a function of pH and is described by an exchange mechanism in which the initial exchange event is protonation of the ring nitrogen (N-1) of adenine. Protonation of N-1 lowers the acid dissociation pK_a of the purine amino from pK_a = 18–19 to pK_a = 10.6–11.7. The range for the latter value represents agreement in the pK_a value determined separately from the same mechanism for three different proton acceptors at different temperatures, *i.e.,* the conjugate bases of imidazole, 2-methylimidazole, and the nucleotide N-1 itself. The experimental pK_a's of the proton acceptors and of the purine N-1 are unique values required for data fitting. From this a novel method is implied for the measurement of (N-1) ligand dissociation constants by the measurement of $-NH_2$ pmr line widths.

The slow intrinsic exchange of amino protons of purines and pyrimidines is more characteristic of the proton exchange of amides, rather than that of aliphatic and aromatic amines (McConnell and Seawell, 1972, 1973; Suchy *et al.,* 1972). This is not surprising, since amide-like character would be associated with amino groups linked to carbon atoms adjacent to electronegative atoms (nitrogen) in the conjugated ring systems of nucleic acid bases. The fact that these amino groups are not titratable in aqueous solution is further testimony of their amide-like character (Christensen *et al.,* 1970). Diffusion controlled (fast) exchange would be expected only for groups that are titratable in water to give "normal" pK_a values (Eigen, 1964).

Although exchange could be initiated by direct protonation of the amino nitrogen, there is evidence that a more complex mechanism is involved. Amines such as imidazole catalyze hydrogen exchange in double helical DNA (McConnell and von Hippel, 1971). Phosphate and imidazole catalyze $-NH_2$ exchange in mononucleotides (McConnell and Seawell, 1972, 1973). As discussed previously (McConnell and Seawell, 1972), direct protonation of the amino nitrogen by H_3O^+ as the predominant rate-limiting event would exclude the ability to observe catalysis by phosphate and amines. Proton transfer theory leads to the prediction that such catalysis would be seen at moderate catalyst concentration only if proton transfer from H_3O^+ to the nucleotide were diffusion controlled (Eigen, 1964). This

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was not the case. It was shown that the catalytic rate constant for H_3O^+ could not exceed 10^7 M^{-1} sec^{-1} in a presumptive direct mechanism (McConnell and Seawell, 1972). Thus, exchange by amines and phosphate indicate that a more complex mechanism could operate, in which the initial proton transfer may be diffusion controlled, but not entirely rate limiting.

On the basis of results obtained from hydrogen exchange studies on polynucleotides Teitelbaum and Englander (1974) present a mechanism in which diffusion limited transfer of a proton from H_3O^+ to adenosine is consistent with slow exchange of the amino protons. Throughout a broad pH range deprotonation of the amino group would occur at observable rates only from the conjugate acid of adenine (protonated at N-1). Although the initial protonation of the base at the ring nitrogen would be diffusion controlled, slow exchange would be seen because the equilibrium concentration of the protonated form is small (pK_a of the nucleotide is 3.5). Under equilibrium conditions of exchange such as those encountered in nuclear magnetic resonance (nmr) experiments, the first-order rate constant for $-NH_2$ exchange should be proportional to (1) the small equilibrium concentration of the protonated nucleotide at the given pH and (2) the rate of removal of the amino proton from the protonated nucleotide by a solvent proton acceptor. Of particular relevance is the finding that a similar exchange mechanism has been proposed as the general case for amides (Martin and Hutton, 1973) and for ureas (Whidby and Morgan, 1973).

$$H^+ + NBNH_2{}^1 \underset{K_a^{nuc}}{\rightleftharpoons} H^+NBNH_2$$

$$H^+NBNH_2 + OH^- \xrightarrow{k_A} H^+NBNH^- + H_2O$$

This study on the catalysis of amino proton exchange by imidazole and 2-methylimidazole supports the proposal that the catalytic mechanism involves binding of the proton to the ring nitrogen (N-1) of adenine. Although the predominant positive agent is the proton, the general case is considered in which the positive agent could be the conjugate acid of imidazole or of the nucleotide itself. In the absence of amine catalyst this mechanism makes little contribution at alkaline pH, where direct deprotonation of $-NH_2$ predominates; or at acid pH, where line broadening is due probably to direct protonation of $-NH_2$ and to decreased rotation about the purine C(6)–N bond. However, the N-1 protonation mechanism is significant in the broad pH region, where line width is relatively invariant with pH. This leads to the novel suggestion that the dissociation constant for binding of positive ions to the basic ring nitrogens of nucleotides can be determined from a single measurement of the $-NH_2$ proton magnetic resonance (pmr) line width. This would represent a simple means for the study of interactions between nucleotides and metal ions or positively charged compounds of biological interest.

Experimental Section

Materials and Methods. Solutions of 2′,3′-cAMP (Sigma) were made without buffer or with stock imidazole solutions prepared from double distilled water. Imidazole (Sigma, Grade III) and 2-methylimidazole (Eastman Organic Chemicals) were recrystallized from benzene and estimated quantitatively in stock solution by pH titration. All measurements of pH were done with a Beckman Model 1019 Research pH meter and are reported as pH values that occur at the temperature of the experiment. Measurements of pH in nucleotide solutions are uniformly higher at 0° by 0.27 ± 0.02 pH unit, as compared to room temperature for the range of pH values used. Results obtained from the pmr spectrometer with pH as a variable were the same if repeated pH adjustments were made on the same solution or if separate solutions of different pH were tested. All solution pH values were rechecked after pmr experiments. The pK_a values for imidazole, 2-methylimidazole and 2′,3′-cAMP, determined in experimental solutions by pH titration, agreed closely with pK_a obtained from pmr chemical shifts. This check was of value in nucleotide solutions at 29°, where the reliability of low pH chemical shifts was in some doubt.

Pmr Procedures. Pmr spectra were obtained under nonsaturating conditions in the Varian HA 100 spectrometer. Chemical shifts were measured relative to Me_4Si (external) enclosed in an inner sealed spherical capillary, which was placed inside the same Wilmad 507-PP Imperial precision bore 5 mm tube for all experiments. Temperature was controlled within ±0.5° with a Varian variable-temperature controller and was monitored by a modified V-6040 Varian temperature control unit that provided continuous digital readout from a probe thermocouple and a Digitec 590TC thermocouple thermometer.

The $-NH_2$ pmr line width of nucleotides under these conditions is a measure of the first-order rate constant for exchange of the amino protons (McConnell and Seawell, 1972; Johnson, 1965). Line-width measurements were averaged from several spectra on the same sample. These spectra were obtained in two ways with respect to the radiofrequency phase adjustment. In one, the radiofrequency phase was adjusted to maximize absorption mode, which provided nucleotide spectra atop a rapidly increasing base line upfield due to the large water absorption. In this case the base-line position under the $-NH_2$ absorbance was established by area measurement (planimeter) of the nonexchanging C-2 and C-8 protons of adenine (McConnell and Seawell, 1972). The established base line was used to replot the $-NH_2$ absorbance for determination of half-height width. In the second, the radiofrequency phase was adjusted to provide a flat water base line, which resulted in a partially dispersion mode $-NH_2$ resonance compressed somewhat on one side and broadened on the other. Line widths of these latter resonances agreed well with those obtained in complete absorption mode, provided that care was taken to account for considerable base line distortion due to another signal in close proximity to the $-NH_2$ resonance. This procedure was a useful check on spectra taken at pH values close to the pK_a of the nucleotide, since the water base line is quite large due to broadening of the water resonance in this pH range.

The pK_a values were determined from plots of chemical shift (relative to Me_4Si) *vs.* pH of the C-2 proton of imidazole, the 2-methyl protons of the substituted imidazole, and the $-NH_2$ or C-2 protons of 2′,3′-cAMP by the relation

$$\delta_{pH} = \frac{\delta_a + \delta_b \times 10^{pH-pK_a}}{1 + 10^{pH-pK_a}} \quad (1)$$

where δ_{pH} is the chemical shift at any pH and δ_a and δ_b are

[1] The nucleotide is abbreviated as $NBNH_2$, where N and NH_2 are the titratable ring nitrogen (N-1) and the amino group, respectively, of the nucleotide base, B. X^+NBNH_2 indicates binding of the positive ion at N-1 of adenine (or N-3 of cytosine). In the text ImH^+ and Im^0 are the conjugate acid and base of imidazole, respectively. K_D^{nuc} and k_A are defined in Results and Discussion.

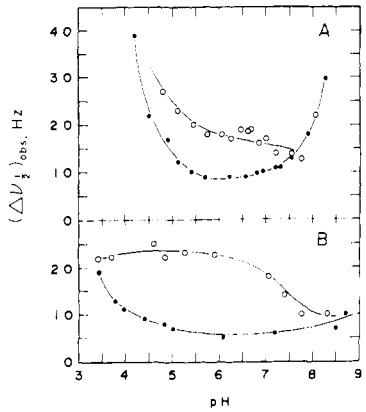


FIGURE 1: The effect of imidazole on the amino pmr line width of aqueous 2′,3′-cAMP. Half-height widths were measured from 100-MHz pmr spectra of 0.13 M solutions of nucleotide in the presence of imidazole (○) and in the absence of imidazole (●). (A) Data obtained at 28 ± 1° and 0.04 M imidazole; (B) data at 0 ● 1° and 0.5 M imidazole. Solid curves are best fits by eye.

chemical shifts corresponding to the conjugate acid (a) and base (b), repectively. In this expression pK_a is the iterated value obtained from curve fitting with the use of the interactive computer. Assumptions implicit in eq 1 are: (1) the uncorrected Henderson–Hasselbalch equation and (2) a linear change in chemical shift with concentration of a given conjugate species. Interactive computer curve fitting for pK determinations and for rate *vs.* pH data was done on the APL/360 computer.

Results and Discussion

Imidazole Catalysis. The $-NH_2$ resonance line widths for 2′,3′-cAMP, plotted as a function of pH for the presence and the absence of imidazole, are illustrated in Figure 1. At both 0 and 29° the addition of imidazole increases exchange in a restricted region of pH. The broadening is maximum and constant below pH 6.8 and falls rapidly to the non-imidazole line width as the pH is raised through the pK_a region of the amine (Figures 1A and B). It is also a function of imidazole concentration (see below). Subtraction of the line widths observed in the absence of added catalyst from the width obtained in its presence isolates the specific catalyst contribution and removes the uncertainties associated with all other broadening factors, including that

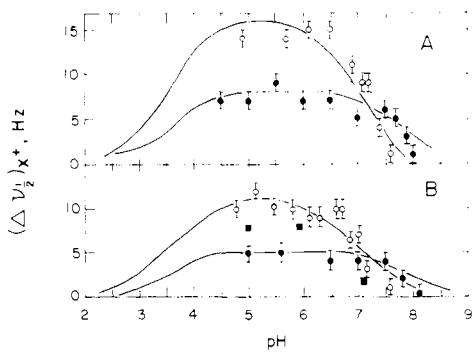


FIGURE 2: The specific broadening of the amino proton resonance of 2′,3′-cAMP at 28° by imidazole and 2-methylimidazole. The values of imidazole broadening, $(\Delta\nu_{1/2})_{X^+}$, were obtained by subtraction of the half-height width in the absence of added catalyst from that obtained in the presence of imidazole (○) and 2-methylimidazole (●). Catalyst concentrations are 0.06 M for A and 0.04 M for B. Nucleotide concentrations are 0.13 M, except for points on B at pH 5.9 and pH 7.1 that correspond to 1 M nucleotide (■). Solid lines through the data are theoretical curves calculated from eq 3 (see text).

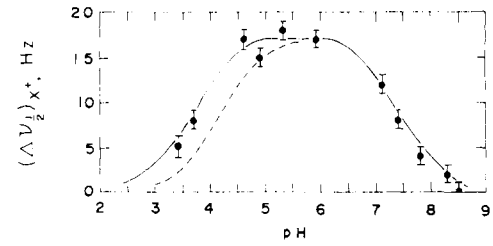


FIGURE 3: Specific imidazole broadening of the $-NH_2$ resonance of 2′,3′-cAMP at 0°. The specific broadening, $(\Delta\nu_{1/2})_{X^+}$, was obtained as for Figure 2 for 0.5 M imidazole, 0.13 M 2′,3′-cAMP solutions at pH values measured at 0°. The raw data are plotted in Figure 1B. The solid line through the points is the theoretical curve calculated from eq 3 and pK_a values of 3.7 and 7.4, for the nucleotide and imidazole, respectively, measured at this temperature. The dotted line is the theoretical curve for an assumed nucleotide pK_a of 4.2 and imidazole pK_a of 7.4.

of intrinsic proton exchange without catalyst. The resulting plots showing specific broadening as a function of pH are shown in Figure 2 for imidazole and 2-methylimidazole and in Figure 3 for imidazole at 0°.

This observation of specific imidazole catalysis can be accounted for by a general scheme, in which the removal of the amino proton from the nucleotide by an acceptor A_i follows the binding of a positive species X^+ to the acidic ring nitrogen of the base, *i.e.*

$$X^+ + NBNH_2{}^1 \underset{K_d}{\rightleftharpoons} X^+NBNH_2 \qquad (2a)$$

$$X^+NBNH_2 + A_i \xrightarrow{k_{A_i}} X^+NBNH^- + H^+A_i \qquad (2b)$$

This leads to the general expression for the specific imidazole broadening and the corresponding first-order rate

$$(\Delta\nu_{1/2})_{X^+} = (1/\pi)k_{X^+} = (1/\pi)\frac{[X^+]\Sigma_i k_{A_i}[A_i]}{K_d + [X^+]} \qquad (3)$$

where $(\Delta\nu_{1/2})_{X^+}$ is the imidazole broadening, k_{X^+} is the first-order rate constant for imidazole catalysis (sec^{-1}) (Johnson, 1965), k_{A_i} is the second-order rate constant for deprotonation of the amino group (M^{-1} sec^{-1}), and K_d (M) is the dissociation constant of the cation–nucleotide complex, X^+NBNH_2.

To account for imidazole catalysis specifically by eq 3, there are a number of possibilities for the identification of X^+ and A_i. If $X^+ = H^+$, then the only possibility for the identity of A_i is the conjugate base of imidazole, but if X^+ is the conjugate acid of imidazole (ImH^+), then there are five additional choices for A_i. These are H_2O, N-1 of adenine, the nucleotide cyclic phosphate group, the ribose C-5′ alcohol, and hydroxyl ion. Their relative strength as proton acceptors is defined by

$$[A_i]k_{A_i} = \frac{k_{diff} \times 10^{pK_{A_i}-pK_D}[A_i]}{1 + 10^{pK_{A_i}-pK_D}} \qquad (4)$$

where $k_{diff.}$ is the diffusion rate constant and pK_{A_i} and pK_D are the acid dissociation pK values of the acceptor and donor, respectively (Eigen, 1964). The donor in this case is the amino group of the X^+–nucleotide complex (eq 2b). A comparison of their relative proton accepting ability is obtained by rearranging eq 4

$$\frac{k_A[A_i]}{k_{diff}} \times 10^{pK_D} = 10^{pK_{A_i}}[A_i]$$

These values are listed in Table I along with their predicted contributions to the observed line width at pH 6.

The strongest of these bases are hydroxyl ion ($pK_a \simeq 16$)

TABLE I: Comparison of Proton Acceptors at pH 6.

Proton Acceptor	Concn of Acceptor Species at pH 6	pK_a (approx.) in Eq 2b	$k_A[A] \times (10^{pK_D}/k_{diff})$	Predicted[a] k_A (M^{-1} sec^{-1}) (eq 4)	Predicted Line Width (Hz) from Eq 3[b] for $X^+ = H^+$
H_2O	55	−2	5.5×10^{-1}	10^{-3}	10^{-4}
Nucleotide $=PO_4^-$	0.15	0.1	1	1	2×10^{-4}
Nucleotide N-1	0.15	3.7	4×10^2	5×10^2	0.8
Imidazole	0.04	7.1	3×10^4	1.3×10^6	7
Nucleotide C(5′)-OH	$<10^{-6}$	>13		10^{10}	<2
Hydroxyl ion	10^{-8}	~16		10^{11}	2–3[c]

[a] From eq 4 for assumed pK_D = 11. The diffusion rate, k_{diff}, is assumed to be 10^{10} for all cases except OH^-, which is measured at 10^{11} M^{-1} sec^{-1} (Eigen, 1964). [b] The use of eq 3 for $X^+ = H^+$ and $K_D = K_a^{nuc}$, the acid dissociation constant of N-1 of 2′,3′-cAMP. [c] $(\Delta\nu_{1/2}) = K_w k_A / K_a^{nuc}$, where K_w is the ion product of water ($10^{-13.8}$ at 29°).

and the ribose C(5′)OH (pK_a > 12) (Sober, 1968). Since the pK_a for each of these is greater (see below) than the pK_a of the H^+NBNH_2 amino group, deprotonation would occur at the diffusion rate (eq 4). Moreover, their contribution without catalyst could be seen in spite of the low concentration of OH^- and the dissociated alcohol (Table I). However, this contribution in imidazole catalysis is relevant only if $X^+ = ImH^+$. Since $[ImH^+]$ is constant at pH values below pH 6.5 the broadening predicted from eq 3 would decrease rapidly below this pH value. This is not observed. The imidazole broadening is constant in the pH range 4.8–6.5 (Figure 2A,B). In addition, the line width is insensitive to large increases in nucleotide concentration in this pH range, which excludes the ribose C′-5 hydroxyl as an important acceptor in the presence or absence of imidazole. Hydroxyl, on the other hand, does contribute when $X^+ = H^+$ (Table I, see below).

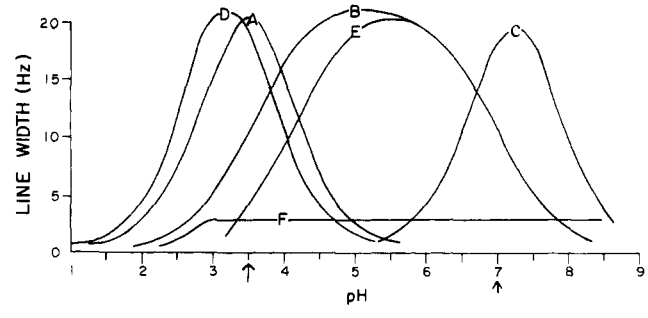
As can be seen in Table I imidazole is the most dominant of the remaining acceptors. Even at pH 5 imidazole would still dominate (4×10^3) in comparison to N-1 of adenine (1×10^2). Therefore the acceptor A_i in eq 3 can be considered the conjugate base of imidazole only, whose concentration is calculated from the total imidazole concentration ([Im]) and the experimentally determined value of its pK_a (from eq 1), *i.e.*

$$[A] = \frac{[Im](10^{pH-pK_a})}{1 + 10^{pH-pK_a}}$$

There are three major possibilities for the selection of the identity of X^+: (1) the proton, H^+; (2) the acid form of the nucleotide (H^+NBNH_2), and (3) the acid form of imidazole[2] (ImH^+). Plots of the calculated variations of line width with pH for these three cases are shown in Figure 4. In Figure 4, curves B and C and E correspond to the imidazole conjugate base (Im^0) as the acceptor, whose pK_a is indicated by the right-hand arrow on the abscissa. For the case $X^+ = H^+$, K_d is the acid dissociation constant of the ring nitrogen of adenine N-1. The pK_a for this was determined experimentally from eq 1 and the pH variation of the C-2 and C-8 proton chemical shifts of adenine in the presence of imidazole. The value obtained (pK_a = 3.5) is required for eq 3 in both the $X^+ = H^+$ and $X^+ = H^+NBNH_2$ cases and is indicated in Figure 4 as the arrow to the left defining a pH region between the pK of the nucleotide and that of imidazole.

It is obvious that a fit of the experimental specific imidazole broadening could be approximated by the first three cases alone (Figure 4, curves B and E) or by a sum of any combination of all three cases. In the case $X^+ = H^+NBNH_2$ two adenine N-1 sites share a proton in a nucleotide–nucleotide complex, which represents the exchangeable species. In this case the rate (and the line width) would be a function of the nucleotide concentration. This would be especially true at the pK_a of the nucleotide. Thus, a significant contribution of this route at pH 5, which approaches the lower limit of measureable line widths at 29°, would be revealed by cAMP-induced broadening. However, a several-fold increase in nucleotide concentration produces no extra increase in line width at this pH or at any higher pH (Figure 2B).

For the case, in which $X^+ = ImH^+$ (curves C or E in Fig-



CURVE	X^+	A	CONDITION
A	H^+	$NBNH_2$	$K_D = K_a^{NUC}$
B	H^+ or H^+NBNH_2	Im^0	$K_D = K_a^{NUC}$
C	ImH^+	Im^0	$K_D \leq [X^+]$
D	H^+NBNH_2	$NBNH_2$	$K_D \sim 0.5$ M
E	ImH^+	Im^0	$K_D \gg [X^+] \sim 30$M
F	H^+	OH^-	

FIGURE 4: Theoretical variations of $-NH_2$ pmr line width with pH conforming to eq 3. The maximum line widths for curves A–E are arbitrarily assigned. Curve F values were obtained from eq 5. Arrows on the abscissa at pH 3.5 and pH 7 locate the pK_a values of 2′,3′-cAMP (N-1) and imidazole, respectively. All curves are identified below the graph for identities of X^+ and A in the equation: line width (Hz) = $[X^+]k_A[A]/\pi(K_d + [A])$ (see text).

[2] Imidazole would not catalyze exchange by donating a proton to the nucleotide base. If we consider proton transfer alone, the concentration of the protonated nucleotide base is established only by the solution pH. A pK_a shift would occur on the addition of protonated imidazole to a solution of the nucleotide, however (see text).

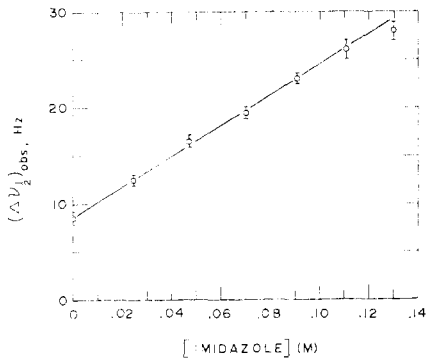


FIGURE 5: The effect of imidazole concentration on the $-NH_2$ pmr line width of 2',3'-cAMP at 29°. Sequential volumes of stock imidazole containing 0.13 M 2',3'-cAMP were added to the same concentration of nucleotide in the same nmr tube for each spectrum. The final pH after each addition of imidazole was pH 6.9.

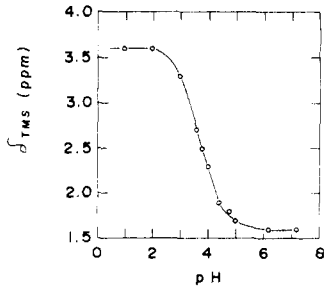


FIGURE 6: Amino pmr chemical shift of 2',3'-cAMP *vs.* pH at 0°. Chemical shifts of the $-NH_2$ proton resonance were measured relative to external Me_4Si for 0.13 M 2',3'-cAMP in the absence of imidazole at pH values measured at 0°. The 0° pK_a value of 3.75 would be corrected to 3.50 at 28°.

ure 4) the line width would increase with the square of the imidazole concentration. For curve E[3] this would be most apparent between pH 5 and pH 6, while it would be most apparent in the pH = pK_a region of imidazole for curve C. A plot of line width *vs.* imidazole concentration at pH 6.9 is linear within experimental error (Figure 5). This linearity is also evident at lower pH values (*cf.* Figure 2A and B). Although the contribution of the $X^+ = ImH^+$ route to the total line width would not exceed 1 Hz, it cannot be excluded entirely as a possible exchange route. For example, it would account nicely for the small difference between the observed and calculated line widths in the vicinity of pH 7 (Figure 2A).

The calculated curves in Figure 2A and B for specific catalysis by the nonsubstituted and substituted imidazoles represent the case for $X^+ = H^+$ and $A = Im^0$. Both the fit of these data and the foregoing arguments suggest that the major exchange route for the amino protons of adenine involves protonation of the nucleotide at N-1 and that the catalytic role of imidazole is limited chiefly to its function as the proton acceptor. For calculation of the theoretical curves the rate constant for $-NH_2$ deprotonation, k_{A_i}, is the only unknown, since K_d is the acid dissociation constant of the nucleotide in this case. The fit for imidazole catalysis illustrated in Figure 2A and B was obtained for $k_{A_i} = 8.4 \pm 0.3 \times 10^5$ M^{-1} Hz (or 2.7×10^6 M^{-1} sec^{-1}) and pK_a values for imidazole and 2',3'-cAMP of 7.0 and 3.5, respectively. This value of k_{A_i} provides an estimate of the pK_a of the amino group of the protonated form of the nucleotide. By rearranging eq 4 and assuming a diffusion rate constant k_{diff} of 10^{10} M^{-1} sec^{-1} a pK_a value of 10.6 is obtained. The accuracy of this pK_a depends most strongly on the assumed value of k_{diff} and on the experimental value of the pK_a of the nucleotide. However, independent measures of this pK_a value for the $-NH_2$ agree quite well when consistent nucleotide pK_a values are used (see below). The theoretical fit for the precipitous drop in the experimental points above pH 7 could be improved and might reflect an abrupt change in the apparent dissociation constants of both imidazole and cAMP as the imidazole is converted rapidly to its uncharged form. The reduction in total charge above pH 7 would suppress the formation of the protonated forms of the nucleotide and the amine, which would correspond to a decrease in the pK_a for each.

The specific catalysis of $-NH_2$ exchange by 2-methylimidazole is shown as a function of pH in Figure 2A and B for two concentrations of the amine. As predicted by eq 3 the amine-induced broadening profile above neutrality is shifted to the higher pH values because of the higher pK_a of the substituted imidazole (pK_a = 7.8). The observed broadening by 2-methylimidazole would be no greater than that induced by an equivalent concentration of unsubstituted imidazole. In fact, the broadening is considerably less for the substituted imidazole. This may reflect the fact that, while the pK_a value of the amine is a function of solvation energy as well as basicity, k_{A_i} is more a function of true basicity of the tertiary nitrogen (Bell and Trotman-Dickensen, 1949). This smaller broadening and the corresponding value of k_{A_i} (8.2×10^6 M^{-1} sec^{-1}) provide a somewhat larger pK_a value for the $-NH_2$ of H^+NBNH_2, *i.e.*, pK_a = 10.9 (pK_D of eq 4).

Although there is good agreement, both in the fit of theoretical and experimental data (Figure 2A and B) and in the kinetic constants, the results derived from these figures alone are not entirely unambiguous with regard to the major exchange routes. For example, the data could be fitted, also, by including a sum of two routes, *i.e.*, $X^+ = ImH^+$, $A = OH^-$ and $X^+ = H^+NBNH_2$, $A = OH^-$. The first of these could account for the fact that 2-methylimidazole is less effective than imidazole. Although the sum of these would show no rate decrease below pH 4 (as does $X^+ = H^+$, $A = ImH^+$), line-width data are inaccessible at 29° for pH values below 4.2 due to rapid line broadening and overlap of the $-NH_2$ resonance with nucleotide and imidazole proton signals. However, sufficiently narrow $-NH_2$ resonances are obtained at 0° for limited acidic pH values selected for minimum signal overlap (Figure 1B). A second advantage of low-temperature spectra is that presumptive contributions involving OH^- should completely disappear because of the large (~10-fold) decrease in K_w between 29 and 0°. This should change the shape of the pH profile at low temperature if the OH^- contribution was large at 29°.

As can be seen in Figure 3, this is not the case. At 0°, the specific broadening by 0.5 M imidazole shows retention of the 29° pH profile characteristics. More important is the fact that the theoretical curve calculated from the measured pK_a of N-1 at 0° tracks through the observed line width at pH 3.7 and pH 3.5. This pK_a value was determined from eq 1 and a plot of the chemical shift of the $-NH_2$ protons *vs.* pH (Figure 6). To illustrate the uniqueness of this pK_a value for use in eq 3, the additional calculated curve in Figure 3 (dotted line) was obtained by readjusting kinetic con-

[3] A condition for this expectation is if $K_D \gg [X^+]$. For a reasonable value of k_A obtained from $X^+ = H^+$, K_d would have to exceed 30 M in order to conform to the observed rates. This number is not unreasonable. Curve C is unlikely, since $K_D \leq [X^+]$ corresponds to an unreasonably low value of k_{A_i} in order to conform to the observed rate.

TABLE II: Apparent pK_a Values Determined from Rate and Titration Data.[a]

Compd	Functional Group	Experimental Method	pK_a[a] Values from $-NH_2$ Exchange 29°	0°
H^+NBNH_2	C-6-NH_2	A = $NBNH_2$ (eq 3)	10.1 ± 0.3[c]	10.8
	C-6-NH_2	A = imidazole (eq 3)	10.6	11.8
	C-6-NH_2	A = 2-methylimidazole (eq 3)	10.9	
$NBNH_2$	C-6-NH_2	Deprotonation by OH^-	18–19[b]	
$NBNH_2$	N-1	pH–titration	3.5	
	N-1	Chemical shift *vs.* pH	3.6	3.7 ± 0.05[d]
	N-1	Line width *vs.* pH (eq 3)		3.7 ± 0.1
Imidazole	N-1,3	pH–titration	7.05	
	N-1,3	Chemical shift *vs.* pH	7.1	7.4 ± 0.1[d]
	N-1,3	Line width *vs.* pH (eq 3)	7.1 ± 0.1	7.4
2-Methyl-imidazole	N-1,3	pH–titration	7.5	
	N-1,3	Chemical shift *vs.* pH	7.8	
	N-1,3	Line width *vs.* pH (eq 3)	7.8 ± 0.1	

[a] pK_a, the negative log of the acid dissociation constant measured under the conditions of the experiments, *i.e.*, 0.15 M 2′,3′-cAMP and 0.04 to 0.06 M imidazole at 29° or 0.5 M imidazole at 0°. [b] Calculated from experimental k_A and eq 4. Assumed values of k_{diff} are 10^{10} M^{-1} sec^{-1} for both 29 and 0°. For OH^- k_{diff} is 10^{11} M^{-1} sec^{-1} (Eigen, 1964). [c] Imidazole not present. [d] Determined with pH measurements measured at 0°, *i.e.*, uncorrected to 29°.

stants to obtain a maximum fit for an assumed pK_a of 4.2 for the nucleotide N-1. This strongly supports the notion of binding of H^+ to N-1 as the initial event in $-NH_2$ exchange. This pK_a and a pK_a of 7.4 for imidazole at 0° provide a pK_a value of 11.7 for the H^+NBNH_2 amino (k_A = 4.2×10^5 M^{-1} sec^{-1} and eq 4). From the 0° data it is most apparent that curve fitting of the catalytic rate data represents an independent measure of the dissociation constant of the X^+-nucleotide complex. In this case it is the acid dissociation constant whose values, determined by different methods, are included in Table II.

Exchange without Imidazole. In the absence of imidazole the line width passes through an 8 to 9 Hz minimum at 29° for a broad range of pH (pH 5.3 to pH 7.3) (Figure 1A). The corresponding curve at 0° (Figure 2) is 5–6 Hz in this pH region and is temperature independent up to a temperature of approximately 10° (unpublished data). Therefore, the 0° minimum line width arises entirely from nonexchange contributions, notably dipolar broadening and quadrupole relaxation of the amino nitrogen. The contribution of the latter could be as much as 3 Hz, but corresponds to such rapid relaxation of the quadrupole moment that attempts to saturate the quadrupole with radiofrequency power from the heteronuclear decoupler do not produce further line narrowing (McConnell and Seawell, 1972). At 29° the quadrupole broadening should not be greater (Roberts, 1956) and should be constant at pH values above the pK of the nucleotide. Therefore, a significant contribution of the minimum line widths at 29° (3–5 Hz) is due to proton exchange and, judging from the 0° line width, at least 4 Hz reflects nonexchange broadening contributions. This 3–5 Hz exchange contribution in the pH region of minimum exchange (between pH 5.5 and 7.2 at 29°) can be accounted for nicely by the mechanism of eq 2, where $X^+ = H^+$ and A = OH^-.[4] In this case eq 3 would become

$$(\Delta\nu_{1/2})_{X^+=H^+,\,A=OH^-} = \left(\frac{1}{\pi}\right)\frac{[H^+]k_A[OH^-]}{K_a^{nuc} + [H^+]} = \left(\frac{1}{\pi}\right)\frac{K_w k_{diff}}{K_a^{nuc}} \quad (5)$$

where K_a^{nuc} is the acid dissociation constant of the nucleotide N-1 (2×10^{-4} M), and K_w is the ion product of water ($10^{-13.8}$ at 29°).

Although the predicted contribution is significant at 2–3 Hz, it is quite invariant with pH (curve F, Figure 4). Such invariance is implicit in eq 3 in the pH region below the pK_a of the acceptor A, which in this case is OH^- ($pK \simeq 16$). The broadening that begins at pH 8 would conform to eq 3 only if a potential cation were present, whose pK_a (or conversion to the cationic form) were in the vicinity of pH 8.5–9.5. Since this is not the case, the alkaline broadening above pH 7.5 (Figure 1A, lower curve) must reflect direct deprotonation of the nucleotide conjugate base $NBNH_2$ by OH^-. This is borne out by the reduced broadening at 0°, where $[OH^-]$ is tenfold less (Figure 1B, lower curve). The second-order rate constant for direct deprotonation, k_{OH^-}, is 7×10^7 M^{-1} sec^{-1} at 29°, which corresponds to a pK_a value of 18.2 or 19.2 for assumed k_{diff} values of 10^{10} and 10^{11} M^{-1} sec^{-1}, respectively (eq 4).

Possible solvent exchange mechanisms to account for the extensive line broadening below pH 5 would include consideration of a direct protonation of the amino group as well as the N-1 protonation mechanism described by eq 2 and 3. Since measurable line widths well into the low pH region are not accessible at 29°, the discussion is restricted momentarily to data obtained at 0°.

Observed $-NH_2$ pmr line widths at 0°, corrected for small pH-induced broadening of nonexchanging protons, are plotted in Figure 7 as a function of pH. In this low pH region the most likely prospects for X^+ and A_i in terms of eq 2 and 3 are $X^+ = H^+$ or H^+NBNH_2 and $A_i = NBNH_2$. Also, the cyclic phosphate group could act as an acceptor in the vicinity of its protonation, which probably occurs below pH 2. However, at this temperature the mechanism of eq 2 and 3 can be rejected as the major factor in low pH line

[4] It is possible that the conjugate base of the nucleotide ribose C(5′)-OH could contribute a constant 1 or 2 Hz throughout the entire pH range at 29°. However, the pK_a of this group is not known and its contribution is not supported by the insensitivity of the minimum line width to large increases in nucleotide concentration.

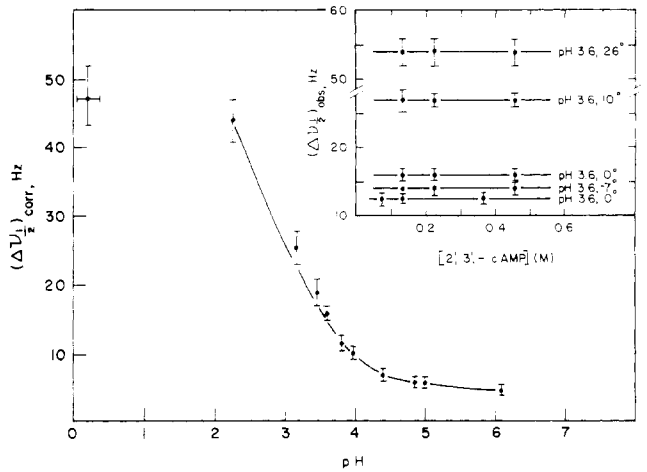


FIGURE 7: Amino pmr line widths of 2′,3′-cAMP at low pH. Line widths, $(\Delta\nu_{1/2})_{corr}$, were obtained at 0° by subtracting the additional broadening of nonexchanging protons from the observed line width. This nonexchange contribution seen below pH 6 by the increase of the H-2 and H-6 resonances did not exceed 2 Hz. Insert: observed line width, $(\Delta\nu_{1/2})_{obsd}$, as a function of 2′,3′-cAMP concentration at various temperatures and pH values. Solid lines through the data are best eye fits.

broadening for the following reasons. First, the exchange route $X^+ = H^+$ and $X^+ = H^+NBNH_2$ (with $A_i = NBNH_2$ for each case) shows a theoretical maximum in the pH = pK_a region of the nucleotide N-1, with the rate *decreasing* at lower pH values. This is illustrated as curve A for $X^+ = H^+$ and curve D for $X^+ = H^+NBNH_2$ in Figure 4. As can be seen in Figure 7 this is clearly not the case. The line broadening below pH 5 continues to increase on the acid side of pH 3.7. Second, if the $-NH_2$ pK_a value, determined for H^+NBNH_2 from imidazole catalysis, is assumed to be correct at 11.8, then the rates predicted from eq 3 and 4 for this identification of X^+ and A correspond to less than 0.5 Hz broadening at the pH maximum. It is quite possible that a lower $-NH_2$ pK_a value would apply due to inaccuracy of the assumed values of k_{diff} for imidazole and the nucleotide. If the pK_a were between 10.8 and 11.8 the small broadening could be accounted for between pH 5 and pH 3.8, but not at the lower pH values. Third, the theoretical variation in line width as a function of nucleotide concentration would be squared for $X^+ = H^+NBNH_2$ and linear for $X^+ = H^+$. However, the *independence* of line width to large concentration changes of the nucleotide is impressive, as can be seen for different pH values and temperature values in Figure 7 (insert).

If direct protonation of the adenine $-NH_2$ were the initial event in exchange, the broadening below pH 4 would be independent of the nucleotide concentrations, but would be expected to increase to invisibility of the $-NH_2$ resonance below pH 3. It is apparent from Figure 7 that this is not the case. The $-NH_2$ resonance is quite visible in 1.5 M H_2SO_4 (pH between 0 and 0.3) with an upper limit line-width measurement of 52 Hz. Also, a significant contribution to line width from processes other than solvent exchange is suggested by the fact that the decrease upon cooling from 0 to −7° is small (Figure 7).

In view of this, the most probable explanation for line broadening of the $-NH_2$ resonance between pH 4 and pH 2 would be decreased $-NH_2$ rotation about the C(6)–N bond in response to protonation of adenine. Decreased $-NH_2$ rotation is seen when cytosine is protonated at its acidic ring nitrogen (N-3) (Miles *et al.*, 1963). This possibility is borne out further by the observation that the amino protons of 2′,3′-cyclic cytosine monophosphate, whose rotation is at the slow pmr exchange limit at 0°, show no broadening of their narrow resonances at any pH below the pK_a of the cytosine N-3 (B. McConnell, in preparation; McConnell and Seawell, 1973). (This also eliminates consideration of the cyclic phosphate group as a factor in exchange broadening at 0° for 2′,3′-cAMP.) The possibility of sufficiently slow rotational exchange of the adenine $-NH_2$ is supported by molecular orbital calculations of Rao and Rao (1973), which indicate that $-NH_2$ rotation of the purine is only slightly greater than that of cytosine. Therefore, at 0° decreased rotation is the most likely line broadening factor below pH 4, while solvent exchange by direct protonation of $-NH_2$ might begin to contribute to the observed line width below pH 1.

In spite of the possible complication of rotational broadening, solvent exchange at low pH is predominant at higher temperature. Because the rotation rate is greater than that required for coalescence of the separate $-NH_2$ proton resonances, an increase in temperature should decrease the $-NH_2$ line width. Instead, an increase in temperature leads to large increases in line width at pH 3.7 (Figure 7), which is still independent of 2′,3′-cAMP concentration at 10 and at 29° (Figure 7). This leads to the suggestion that the N-1 protonation scheme of eq 2 and 3 may be a less prominent route than direct protonation of $-NH_2$ for low pH exchange at 10 and 29°. A more definitive assessment of the relative low pH contribution of rotation, direct protonation, and the mechanism of eq 2 will require accurate line width measurements at several temperatures between −20 and +10°.

Conclusions and Implications

For a broad pH range near neutrality the major exchange route for the $-NH_2$ protons of adenine nucleotides involves the diffusion controlled transfer of a solvent proton to the titratable *ring nitrogen* of the purine. This conclusion is based on (1) agreement between theoretical and experimental pH–rate profiles for imadazole catalysis, (2) the extraction of the known pK_a of the adenine N-1 from the appropriate rate equation, and (3) the agreement in the pK_a values of $-NH_2$ of the active nucleotide intermediate H^+NBNH_2 that were obtained independently from rate constants for $-NH_2$ deprotonation by imidazole and 2-methylimidazole. Tentative values for this latter pK_a obtained in the absence of imidazole where the acceptor is the conjugate base of the nucleotide agree with these values within 1 pK_a unit. Other evidence yet to be presented for such a general mechanism are the observations that phosphate can replace imidazole as the acceptor to achieve the same result, that a metal ion (*e.g.*, methylmercury I) can catalyze the exchange of $-NH_2$ protons and that exchange of the $-NH_2$ protons of cytosine and guanine conform to eq 3 (B. McConnell *et al.*, in preparation).

In the absence of imidazole this N-1 protonation mechanism appears to be significant only in the broad pH region where $-NH_2$ pmr line width is invariant with pH. An important implication of this is that a positive entity of any source could broaden the $-NH_2$ resonance by its binding to the basic ring nitrogen of a purine or pyrimidine. Thus, if k_{A_i} in eq 2 were known, the line width would be a quantitative measure of K_d, the dissociation constant of the X^+-nucleotide complex. In fact, k_{A_i} is indeed known regardless of the nature of the positive ion. This is because the most important acceptor in the minimum pH range is OH^-.

Since the pK of OH^- would be larger than the pK_a of the amino group of the X^+-nucleotide complex the observed broadening by ligand binding would be insensitive to changes in this $-NH_2$ pK_a value, which might be associated with the particular nature of the cation. This would represent a distinct advantage over other nucleotide–cation binding studies because there is no need to saturate the nucleotide with cation to obtain measurable spectral changes. The $-NH_2$ line width is sensitive to a small equilibrium concentration of the complex and would provide the X^+-nucleotide dissociation constant in one measurement. Several potential cations are attractive candidates for studying interactions at the acidic ring nitrogen of nucleotides and possibly polynucleotides. Some of these, in addition to metal cations, might be active forms of mutagenic carcinogens, particularly those whose activity can be associated with an incipient or formal positive charge that can lead to substitution of the basic ring nitrogens (Irving, 1973).

Acknowledgments

I wish to thank Pat Seawell for her excellent technical assistance in the exploratory aspects of this work and Mrs. Katherine Cramer for her skillful assistance in the collection of the bulk of the pmr data.

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Chromophoric Labeling of Yeast 3-Phosphoglycerate Kinase with an Organomercurial†

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ABSTRACT: Phosphoglycerate kinase, purified and crystallized from yeast, was reacted with the organomercurial, 2-chloromercuri-4-nitrophenol. The reaction products whether formed with the native enzyme or with enzyme denatured by 5 M urea or by 3.5 mM sodium dodecyl sulfate, followed by renaturation, appeared identical and were fully active. The modified enzyme appeared identical with the native enzyme in its catalytic properties. Only 1 mol of mercurial or 1 mol of 5,5′-dithiobis(2-nitrobenzoic acid) reacted per mol of enzyme. The pK_a of the phenolic hydroxyl of the free mercurial is 6.60 but became 8.30 when the compound associated with the single thiol residue of the enzyme. This association also resulted in shifts in the wavelength of maximum extinction (red shift) and decreased the extinction at these maxima (hypochromicity) for the acid and base forms of the mercurial. The slower electrophoretic mobility of the modified enzyme was readily reversed by β-mercaptoethanol. The mercurial dissociated from the enzyme during polyacrylamide gel isoelectric focusing to yield an isoelectric point of 7.01, identical with that of native enzyme. Interactions between the mercurial and enzyme were exemplified in a skewed titration curve and a slope between 1 and 2 for the linear representation of the Henderson–Hasselbalch equation. The phenolic hydroxyl of the mercurial bound to phosphoglycerate kinase had a pK_a of 8.72 in the presence of the substrate 3-phosphoglycerate and 8.54 in the presence of $MgATP^{2-}$. The rate of reaction between the mercurial and the enzyme is increased by $MgADP^-$ and $MgATP^{2-}$ and is decreased by 3-phosphoglycerate and phosphate. This reaction in a mixture of $MgADP^-$ and 3-phosphoglycerate had a rate intermediate between the rates in the two substrates added separately. The relevance of these findings to the mechanism of the enzyme is discussed.

Phosphoglycerate kinase (EC 2.7.2.3) is an important enzyme in the glycolytic pathway in that the reaction it catalyzes provides much of the driving force for the preceding steps in the sequence and particularly for the glyceraldehyde-3-phosphate dehydrogenase reaction (Mahler and Cordes, 1966). Until recently, investigations concerning this enzyme lagged well behind those on other glycolytic enzymes. The enzyme is the only monomer in the glycolytic pathway and has a molecular weight of 47,000 (see Scopes,

† From the Department of Pathology, Division of Medical Laboratory Science, University of Alberta, Edmonton, Canada. *Received May 7, 1974.* This work was supported by the Medical Research Council of Canada. A preliminary report has been presented (Stinson, 1972).