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Proton Nuclear Magnetic Resonance Study on Uridine Imido Proton Exchange[†]

H. Fritzsche,[‡] L.-S. Kan,* and P. O. P. Ts'o

ABSTRACT: The exchange of the N(3)-H imido proton of uridine has been studied in aqueous solution by ¹H NMR as a function of pH and temperature. At temperatures ranging from -1 to +30 °C, the minimum exchange rate is found at pH 3.4-4.2. The exchange rate as a function of pH has been interpreted in terms of general base-acid catalysis. The proton-catalyzed exchange rates, $k_{\text{H}_3\text{O}^+}$, are in the order of $10^4 \text{ M}^{-1} \text{ s}^{-1}$ but the base-catalyzed exchange rates, k_{OH^-} , are in the order of $10^{10} \text{ M}^{-1} \text{ s}^{-1}$, typical for diffusion-limited processes. The thermodynamic activation parameters from pH 1.7 to pH 5.3 have been evaluated from the temperature dependence of the exchange rate to be $\Delta H^{\ddagger} = 7.8 \pm 0.8 \text{ kcal M}^{-1}$, $\Delta S^{\ddagger} = -22.6 \pm 1.8 \text{ eu}$, and $\Delta G^{\ddagger} = 14.5 \pm 0.6 \text{ kcal M}^{-1}$. The existence of a different predominant exchange mechanism at below pH 5.3 is indicated by a decrease of the activation enthalpy ($\Delta H^{\ddagger} \simeq 4.0 \text{ kcal M}^{-1}$) accompanied by a drop of the entropy ($\Delta S^{\ddagger} \simeq -32.5 \text{ eu}$); the activation free enthalpy

ΔG^{\ddagger} is not significantly affected by the change of the exchange mechanism. The catalytic effect on the NH exchange rate at pH_{min} (3.7 ± 0.1) has been studied as a function of concentration of the catalysts, phosphate and trifluoroethylamine (TFEA). At 20 °C, the enhancement of the exchange rate by the basic form of phosphate and TFEA is characterized by $k_{\text{cat}} = 1738$ and $2.45 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$, respectively, when k_{cat} 's are derived from the total concentration of the catalyst, or, taking into account the actual concentration of the basic form of phosphate and TFEA at pH 3.7-3.8, $k_{\text{cat}} = 7.0 \times 10^4$ and $2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, respectively. These values are in excellent agreement with the calculated rates according to Eigen's theory of proton transfer processes. Therefore, the phosphate and TFEA can be considered two catalysts with their relative catalytic strength proportionally relative to their respective proton acceptor strength.

The exchange rate of the NH, NH₂, and OH protons of nucleic acids has been widely studied to obtain structural and conformational information (Printz & Von Hippel, 1965; Englander & Englander, 1965; Englander, S. W., et al., 1972; Englander, J. J., et al., 1972; Englander & Von Hippel, 1972). Estimation of the number of slow exchangeable protons of nucleic acids provides a description of the tertiary structure of these molecules in solution (Englander & Englander, 1965). Furthermore, the exchange behavior reflects the time-dependent conformational fluctuations ("breathing") of the secondary and tertiary structure of nucleic acids (Printz &

Von Hippel, 1965; Englander & Englander, 1965).

Pioneering work (Printz & Von Hippel, 1965; Englander & Englander, 1965) has been done by the gel filtration technique monitoring the tritium-hydrogen reexchange as a function of time (Englander & Englander, 1978). The corresponding deuterium-hydrogen reexchange has been monitored by several spectroscopic methods such as real-time nuclear magnetic resonance (Johnston & Redfield, 1979) and the stopped-flow technique in the ultraviolet region (Cross, 1975; Nakanishi et al., 1977; Nakanishi & Tsuboi, 1978). The time scale has been expanded by nuclear magnetic resonance techniques in the frequency domain (Marshall & Grunwald, 1969) and in the time domain (Johnston & Redfield, 1979), measuring the line width and the relaxation time, respectively.

Fundamental interpretation of the exchange processes of nucleic acid base protons in ordered structures is based upon the knowledge of the exchange kinetics of the mononucleotides or nucleosides. The NH₂ exchange of adenine, guanine, and cytosine nucleotides has been studied thoroughly (McConnell

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& Seawell, 1972; McConnell et al., 1972; Cross, 1975; McConnell, 1978), while the exchange processes of the NH protons of the nucleosides or nucleotides have been studied only on two compounds—guanosine cyclic 2',3'-monophosphate (McConnell, 1978) and uridine (20 °C) (Mandal et al., 1979). However, the chemical shifts of the imido protons of the A·U and the imido protons of the G·C base pairs of nucleic acids have been the subject of numerous NMR studies [cf. the review of Kearns (1976)].

In this paper, we present a complete study of the N(3)-H exchange of uridine in aqueous solution as a function of both pH and temperature. All thermodynamical parameters of the exchange process have been calculated. The influence of phosphate and trifluoroethylamine (TFEA) as exchange catalysts has been investigated at the pH where the minimum exchange rate occurs. The catalytic rate of these two catalysts has been measured, calculated, and compared.

Experimental Procedures

Uridine (from Sigma Chemical Co., St. Louis, MO) has been dissolved in a mixture of 80% H₂O and 20% D₂O. The deuterium oxide provided the lock signal in the NMR experiment. All water used in the experiments was doubly deionized and sterilized. Purification of uridine by passing through a chelex column was without significant influence on the results. 2,2,2-Trifluoroethylamine hydrochloride (TFEA) was from Aldrich Chemical Co.

The ¹H NMR spectra were recorded on a Bruker WH-360 spectrometer, which is located at Mid-Atlantic NMR Facility Center, University of Pennsylvania, Philadelphia, PA. A special program has been used to correct the distortion by fast sweeping through a small frequency range (Dadok & Sprecher, 1974). The variable-temperature accessory (accuracy ≈ 1 °C) has been used in the range of -1 to +30 °C.

The pH has been controlled by a Sargent-Welch NX pH meter equipped with an Ingold combination microelectrode. pH was measured inside the NMR tube before and after the NMR experiment.

In order to check any frequency dependence of the line width of N(3)-H of uridine, three other NMR spectrometers were used: (1) a Varian A360, 60 MHz, operated with continuous wave mode, located at National Institutes of Health, Bethesda, MD; (2) a Varian XL-200, 200 MHz, operated with FT mode, located at Varian Associates, Florham Park, NJ; (3) a home-built 500-MHz NMR spectrometer located at the Francis Bitter National Magnet Laboratory, Cambridge, MA.

Results

Uridine. The NMR line width of 0.2 M aqueous solution of uridine at ca. 11.0–11.3 ppm [N(3)-H] has been measured as a function of pH at -1, 10, 20, and 30 °C (Figure 1). The exchange process produces an excess line width $\Delta\nu_{1/2}^e$ which has been determined by subtracting the line width of the nonexchangeable uridine H(6) proton (the doublet at ca. 7.8 ppm) from the measured line width $\Delta\nu_{1/2}^{\text{NH}}$:

$$\Delta\nu_{1/2}^e = \Delta\nu_{1/2}^{\text{NH}} - \Delta\nu_{1/2}^{\text{H(6)}} \quad (1)$$

This procedure also accounts for the artificial line broadening which may be produced by the inhomogeneous magnetic field.

The dependency of the line width at half-height of the uridine N(3)-H resonance on NMR frequency was examined by observing a 0.2 M, pH 3.7 uridine sample (no buffer added) from 60 to 500 MHz at 0 °C. The observed line widths ($\Delta\nu_{1/2}^e$) of N(3)-H are 11.2, 11.5, 12.5, and 13.3 from the 60-, 200-, 360-, and 500-MHz NMR spectrometers, respec-

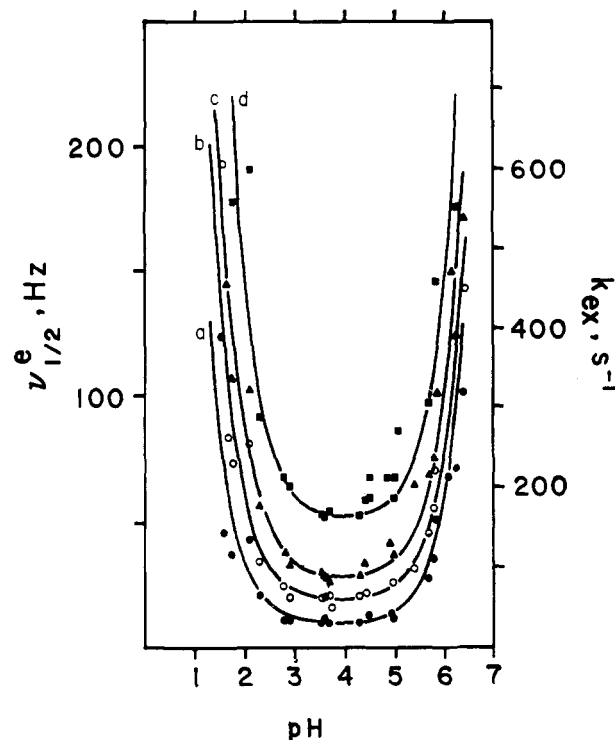


FIGURE 1: Excess line width $\Delta\nu_{1/2}^e$ (in Hz) of uridine N(3)-H NMR line in 0.2 M aqueous solution as a function of pH. Right coordinate scale: exchange rate $k_{\text{ex}} = \pi(\Delta\nu_{1/2}^e)^2$ (in M⁻¹ s⁻¹). (a) -1; (b) 10; (c) 20; (d) 30 °C. Circles: observed values; solid lines: calculated by eq 3 and 4 with the parameters of Table I.

tively. These results showed that the line widths of the uridine N(3)-H proton resonance are independent of the frequency of the NMR spectrometer from 60 to 500 MHz. Therefore, the exchange rate of N(3)-H of uridine can be considered as "slow" with respect to the NMR frequency. Similar results were observed by McConnell & Seawell (1972) in their NMR study of the proton exchange of adenine and guanine nucleotides.

We have also examined the influence of the uridine concentration on the exchange rate in order to evaluate the possible effect of self-catalysis. The measured exchange rates of both 0.05 and 0.1 M uridine are within the limits of error equal to the corresponding values of 0.2 M uridine at the pH range 3.7–4.9.

In the slow exchange limit, the specific exchange rate is connected with the excess line width by

$$\Delta\nu_{1/2}^e = \frac{1-p}{\pi} \left(\frac{1}{\tau} \right)_{\text{NH}} \quad (2)$$

where p is the mole fraction of imido protons, i.e., $p = [\text{NH}]/([\text{NH}] + [\text{H}_2\text{O}])$ and $1-p \approx 1$, and $(1/\tau)_{\text{NH}}$ is the reciprocal of the proton lifetime on the imido nitrogen, i.e., the pseudo-first-order rate constant k_{ex} (Meiboom, 1960). The exchange rate can be split in two terms:

$$\pi(\Delta\nu_{1/2}^e) \approx (1/\tau)_{\text{NH}} = (1/\tau)_{\text{ex}} + (1/\tau)_{\text{pH}} \quad (3)$$

The first term $(1/\tau)_{\text{ex}}$ is the specific exchange rate from catalysis by solution components other than H₃O⁺ and OH⁻; the second term $(1/\tau)_{\text{pH}}$ represents the exchange initiated by H₃O⁺ and OH⁻ and can be described by the relation (McConnell & Seawell, 1972)

$$(1/\tau)_{\text{pH}} = k_{\text{H}_3\text{O}^+} a_{\text{H}_3\text{O}^+} + k_{\text{OH}^-} (K_w/a_{\text{H}_3\text{O}^+}) \quad (4)$$

where $k_{\text{H}_3\text{O}^+}$ and k_{OH^-} are the second-order rate constants for H₃O⁺ and OH⁻ catalysis, respectively, $a_{\text{H}_3\text{O}^+}$ is the H₃O⁺ ac-

Table I: Kinetic Parameters of the NH Hydrogen Exchange of Some Nucleic Acid Bases

sample	T (°C)	(1/τ) _{ex} (s ⁻¹)	k _{H₃O⁺} (M ⁻¹ s ⁻¹)	k _{OH⁻} (M ⁻¹ s ⁻¹)	k _{cat} [A] ⁻¹ (s ⁻¹)			pH _{min}
					phosphate	TEFA	imidazole	
uridine (0.2 M)	-1	31	0.74 × 10 ⁴	1.5 × 10 ¹⁰	2.5 × 10 ⁴	1.02 × 10 ⁶		3.8
	5				3.3 × 10 ⁴			3.7
	10	60	1.25 × 10 ⁴	2.0 × 10 ¹⁰	4.0 × 10 ⁴	1.72 × 10 ⁶		3.9
	20	85	2.0 × 10 ⁴	2.5 × 10 ¹⁰	7.0 × 10 ⁴	1.95 × 10 ⁶		3.9
	30	160	3.0 × 10 ⁴	3.0 × 10 ¹⁰	12.0 × 10 ⁴	2.48 × 10 ⁶		4.0
uridine (0.1 M) ^a	20	130	1.3 × 10 ⁴	1.5 × 10 ¹⁰			1 × 10 ⁷	3.9
5'-AMP ^b	28	69	3 × 10 ⁸	2.5 × 10 ⁷				7.7
5'-GMP (0.1 M) ^c	30	38		5 × 10 ⁸				5-6
purine (0.04-0.85) ^d	20		(2-3) × 10 ¹⁰					6.4

^a Mandal et al. (1979). ^b McConnell et al. (1972). ^c McConnell & Seawell (1972). ^d Marshall & Grunwald (1969).

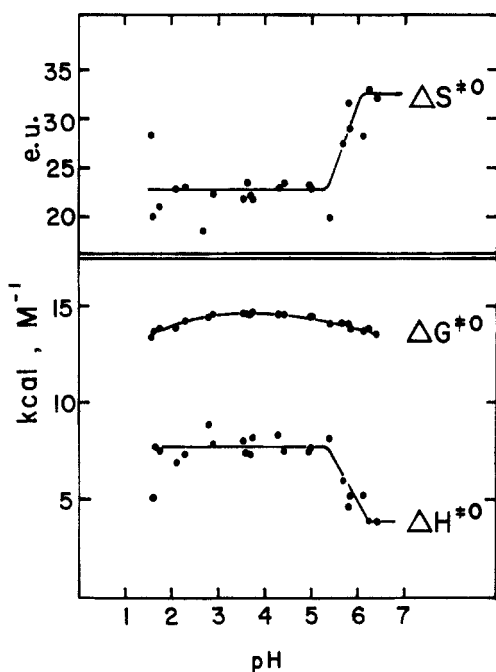


FIGURE 2: Plot of activation parameters $\Delta H^{\ddagger\circ}$, $\Delta G^{\ddagger\circ}$, and $\Delta S^{\ddagger\circ}$ of uridine N(3)-H proton exchange vs. pH.

tivity obtained from pH readings, and K_w is the ion product of water. Equations 3 and 4 have been used to estimate $k_{H_3O^+}$ and k_{OH^-} by fitting assumed values of these two rate constants to the observed dependence of $\Delta\nu_{1/2}^e$ from pH. The numeric values for the best fit of the data are listed in Table I. The lines in Figure 1 are drawn by using the values of Table I to calculate $\Delta\nu_{1/2}^e$ according to eq 3 and 4. The pH minimum for the exchange rate is found at about pH 3.7-4.1. The pH minimum of the uridine exchange is far from the values of the NH_2 exchange of purine bases (5'-AMP, 5'-GMP, and purine) found at pH 5-7.7 (Table I). Moreover, k_{OH^-} of the uridine exchange is in the order of diffusion-limited processes ($10^{10} M^{-1} s^{-1}$) in contrast to the purine base NH_2 with k_{OH^-} in the order of 10^7 - $10^8 M^{-1} s^{-1}$ (Table I). The anion form of uridine may contribute an "additional" mechanism of the line broadening effect on the uridine NH resonance at high pH. However, the pH-dependent experiment was terminated at pH ~ 6.5 , where the NH line width becomes too broad for measurement (Figure 1). The concentration of anion form of uridine will be $(10^{9.8-6.5})^{-1}$ or 0.05% of total uridine concentration at pH 6.5. Therefore, the possible contribution of anionic uridine to the NH resonance is insignificant.

The temperature dependence of the apparent exchange rate $k_{ex} = \pi(\Delta\nu_{1/2}^e)$ has been analyzed by the Eyrings theory for the calculation of the activation enthalpy $\Delta H^{\ddagger\circ}$, the free enthalpy $\Delta G^{\ddagger\circ}$, and the entropy $\Delta S^{\ddagger\circ}$ of the exchange process.

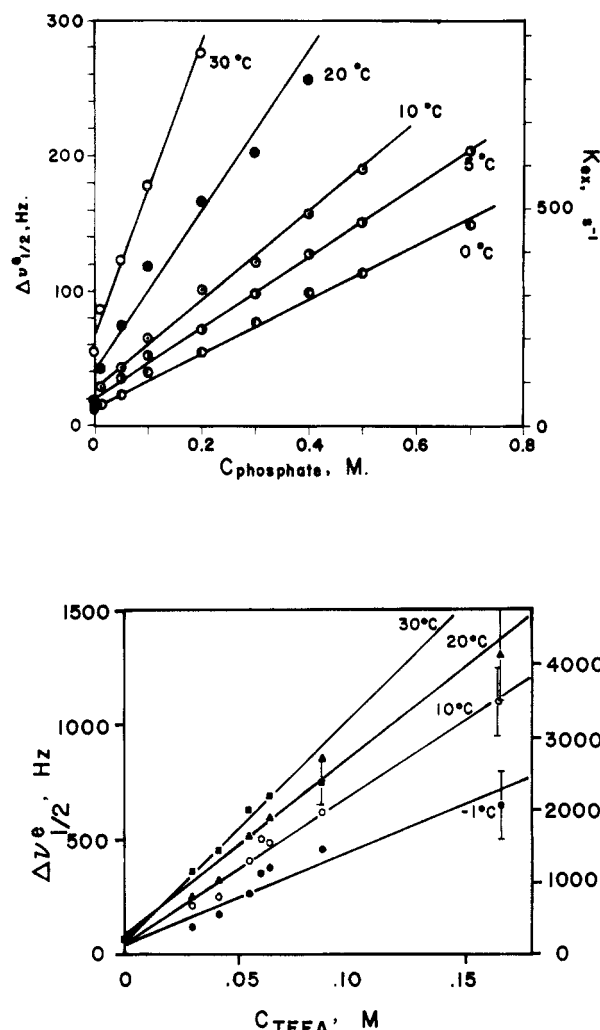


FIGURE 3: 1H NMR excess line width $\Delta\nu_{1/2}^e$ (in Hz) and exchange rate $k_{ex} = \pi(\Delta\nu_{1/2}^e)$ (in s^{-1}) of 0.2 M uridine, pH 3.7 (± 0.1), as a function of concentration of the catalysts (in M): phosphate (top) and 2,2,2-trifluoroethylamine (TFEA) (bottom).

The dependence of these values from pH is shown in Figure 2. Both $\Delta H^{\ddagger\circ}$ and $\Delta S^{\ddagger\circ}$ have a sharp transition at ca. pH 5.6. $\Delta H^{\ddagger\circ}$ drops down from 7.8 to 4.0 kcal M^{-1} and $\Delta S^{\ddagger\circ}$ changes from -22.6 to -32.5 eu.

Uridine Exchange in the Presence of the Catalysts Phosphate and Trifluoroethylamine. The influence of a catalyst on the minimum exchange rate of the N(3)-H proton of uridine at pH 3.7 (± 0.1) has been studied at -1, 5, 10, 20, and 30 °C. The catalysts phosphate and trifluoroethylamine (TFEA) have been added up to 0.7 and 0.166 M, respectively (Figure 3). Assuming a linear enhancement of the exchange rate as a function of the catalysts' concentration, the influence

Table II: Thermodynamic Parameters of Hydrogen Exchange of Uridine NH Protons (25 °C)

sample	$\Delta H^{\ddagger 0}$ (kcal M ⁻¹)	$\Delta S^{\ddagger 0}$ (eu)	$\Delta G^{\ddagger 0}$ (kcal M ⁻¹)
uridine, pH <5.3	7.8 ± 0.8 ^a	-22.6 ± 1.8 ^a	14.5 ± 0.6 ^a
uridine, pH >5.3	4.0	-32.5	13.6
uridine ^b	12.3		
poly(uridylic acid) ^b	18.5		
poly(adenylic acid)- poly(uridylic acid) ^c	15	-7.5	(17.2)

^a Standard deviation. ^b Young & Kallenbach (1978). ^c Mandal et al. (1979).

of the catalyst can be described by the slope of the least-squares lines of Figure 3. The slope is identified as the catalytic rate constant k_{cat} and has been included in Table I.

Discussion

General Acid-Base Catalyzed NH Exchange of Uridine.

The exchange of the N(3)-H imido proton of uridine is catalyzed by OH⁻ with a rate constant $k_{\text{OH}^-} = (1.5-3.0) \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$ in the temperature range of -1 to 30 °C (Table I). This value is expected for a diffusion-limited exchange process (Eigen, 1964). On the other hand, the rate constant, $k_{\text{H}_3\text{O}^+}$, of the acid catalysis is in the order of $10^4 \text{ M}^{-1} \text{ s}^{-1}$ (Table I), indicating the existence of another limiting process. This process may be a second protonation of N(3) as suggested by Mandal et al. (1979). Around pH 4 the exchange is pH independent with a rate constant k_{ex} of 31 to 160 s⁻¹ at -1 to 30 °C, respectively (Table I).

Our results are very similar to those reported by Mandal et al. (1979) at 20 °C; they found, however, a higher value of k_{ex} , probably related to the different line widths of N(3)-H resonance of uridine, 44 Hz from Mandal et al. (1979) and 29 Hz from this work, also at 20 °C. This difference may come from different sample conditions.

The most surprising result is the dramatic decrease of both the activation of enthalpy $\Delta H^{\ddagger 0}$ and the activation entropy $\Delta S^{\ddagger 0}$ at pH >5.3 (Figure 2). The activation enthalpy has not been measured previously and was assumed to be constant and about 14 kcal (Mandal et al., 1979). The free enthalpy of the activation, $\Delta G^{\ddagger 0}$, however, is not significantly changed in this pH region (Table II). In addition, the $\Delta H^{\ddagger 0}$ value of the uridine NH exchange in uridine and in poly(uridylic acid) published by Young & Kallenbach (1978) differs considerably from ours (Table II). From the literature values, the activation enthalpy seems to increase from the mononucleotide to the single-stranded polynucleotide and the double-stranded polynucleotide complex (Table II). This is compensated by a decrease of the (negative) activation entropy.

The sharp drop of $\Delta H^{\ddagger 0}$ at pH 5.3 indicates an abrupt change of the exchange mechanism. A possible explanation may be given by the assumption of a further limiting process (preequilibrium) at pH <5.3. One may propose a water-catalyzed pathway in the pH-insensitive region at pH <5.3 as postulated by Mandal et al. (1979).

Exchange Catalysis by Phosphate and Trifluoroethylamine (TFEA). The catalysis of uridine imido proton exchange by phosphate or TFEA is described by the general equation



where BH is the nucleoside uridine and A and HA⁺ are the conjugate species of the exchange catalyst. The proton donor uridine is characterized by its $\text{pK}_{\text{D}} = 9.5$ and the proton ac-

ceptors by their pK_{A} 's (phosphate $\text{pK}_{\text{A}} = 2.1$ and TFEA $\text{pK}_{\text{A}} = 5.6$). According to Eigen (1964), the exchange rate of proton transfer processes is determined by the pK difference of acceptor and donor:

$$k_{\text{ex}} = k_{\text{d}}[\text{A}](10^{\text{pK}_{\text{A}}-\text{pK}_{\text{D}}})/(1 + 10^{\text{pK}_{\text{A}}-\text{pK}_{\text{D}}}) \quad (6)$$

where k_{d} is the diffusion-limited collision rate (20 °C: $1.5 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$) and [A] is the concentration of the catalyzing species (phosphate or TFEA). The value of k_{cat} in eq 5, which is the same k_{ex} from eq 6, becomes $k_{\text{cat}} = 1.5 \times 10^{10} \times 10^{5.6-9.5}[\text{A}] \text{ s}^{-1} = 10^{6.3}[\text{A}] \text{ s}^{-1}$. On the other hand, the experimental value (Figure 3) at 20 °C yields $2.45 \times 10^4 [\text{cat.}]$ where [cat.] is the total concentration of the catalyst TFEA. At pH 3.7, the actual concentration of catalyzing species A can be calculated by the factor $1/10^{3.7-5.6} = 79.4$. Thus, $k_{\text{cat}} = 2.45 \times 10^4 \times 79.4[\text{A}] \text{ s}^{-1} = 10^{6.3}[\text{A}] \text{ s}^{-1}$. Thus, the experimental value is in excellent agreement to the calculated value. Similar calculation on phosphate was also done. Again, the experimental k_{cat} of phosphate matches the calculated value. The overall exchange rate, induced by H_3O^+ , OH⁻, and the catalyst A, is given by

$$k_{\text{ex}} = k_0 + k_{\text{H}_3\text{O}^+}[\text{H}_3\text{O}^+] + k_{\text{OH}^-}(K_{\text{w}}/[\text{H}_3\text{O}^+]) + k_{\text{HA}^+}[\text{HA}^+] + k_{\text{A}}[\text{A}] \quad (7)$$

where k_0 is the rate unaffected by H_3O^+ , OH⁻, and the catalyst. As can be estimated, the predominant term of eq 7 at pH_{min} (3.7) is $k_{\text{A}}[\text{A}]$; all the other terms are negligible.

The results demonstrate that the exchange catalysis can be described completely by Eigen's theory of proton transfer simple systems. This is in accord with results of Mandal et al. (1979) on the exchange catalysis of uridine by imidazole ($\text{pK}_{\text{A}} = 7.1$); they found $k_{\text{cat}} = 1 \times 10^7 [\text{Im}] \text{ s}^{-1}$, in close agreement to the expected value $1.5 \times 10^{10} \times 10^{7.1-9.5} = 10^{7.8}$.

Conclusions

(1) The hydrogen exchange of N(3)-H imino protons of uridine is characterized by a minimum of the exchange rate located at pH 3.5-4.2. At higher pH, the exchange is catalyzed by OH⁻ at a diffusion-limited rate ($\approx 10^{10}$). At lower pH, the H_3O^+ -catalyzed reaction is drastically slower ($\approx 10^4$). The pH-independent water-catalyzed exchange (pH 3.5-4.2) takes place with a rate of ca. 30-160 s⁻¹ (-1 to 30 °C).

(2) The activation parameters indicate a change of the exchange mechanism of uridine at pH 5.3 by a decrease of enthalpy and entropy at constant free enthalpy of activation. This can be interpreted as transition from water-catalyzed slow exchange to very fast (diffusion-limited) OH⁻-catalyzed exchange.

(3) The enhancement of the slow exchange of uridine at pH 3.7 (± 0.1) by the two catalysts of different strength phosphate and trifluoroethylamine was measured. The experimental values are in excellent agreement with calculated values on the basis of Eigen's theory of proton transfer.

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and home-built 500-MHz NMR spectrometers. Finally, we also thank Man-Nie Kuo for her excellent technical help.

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Kinetic Evidence for Active Monomers during the Reassembly of Denatured Creatine Kinase[†]

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ABSTRACT: Treatment of rabbit muscle creatine kinase, which normally exists as a dimer, with various denaturants leads to different states of unfolded protein. Studies of intrinsic fluorescence indicate the degree of denaturation is most pronounced with 8 M urea, followed by 4 M guanidine hydrochloride and then 1 M glycine-H₂PO₄, pH 2.3, buffer. Titration of creatine kinase with increasing concentrations of urea produces parallel changes in inactivation, denaturation (as measured by fluorescence changes and reacting sulfhydryl groups), and dissociation. Renaturation, achieved by dialysis or dilution, leads to 70% recovery of activity. The remainder is in the form of high molecular weight, inactive aggregates. The kinetics of reactivation of creatine kinase denatured in 8 M urea indicates that the rate and percent of reactivation are independent of enzyme concentration ($k_1 = 1.7 \times 10^{-3} \text{ s}^{-1}$, at 22 °C). The renaturation rate observed by measuring the decrease in intrinsic fluorescence is also independent of enzyme

concentration. Here the kinetic profile exhibits a phase with a rate constant similar to that found for reactivation. Changes in fluorescence are complete after 30 min of initiating renaturation. Renaturation of creatine kinase treated with different denaturants exhibits very similar kinetic profiles and rate constants. Reassociation, as determined by competitive dimerization and hybridization followed by electrophoretic separation or by a selective ultrafiltration technique, reveals that dimerization ($k_2 = 1.8 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$) is still occurring after renaturation and reactivation are complete. The concentration independence of reactivation considered along with the kinetics and temporal relationship among reactivation, renaturation, and reassociation leads to the conclusion that the monomeric subunits of creatine kinase are active. Apparently dimerization is not an obligatory requirement for the expression of enzymic activity.

Numerous studies dealing with the reversibility of protein denaturation have demonstrated that the folding of a polypeptide chain into its native conformation is directly and only dependent upon its amino acid sequence and aqueous environment (Tanford, 1968; Wetlaufer & Ristow, 1973; Anfinsen & Scheraga, 1975). Oligomeric proteins also possess the information in their subunit structure to determine highly specific associations (Cook & Koshland, 1969; Teipel &

Koshland, 1971a,b). The importance of association to enzyme function in vivo is based upon whether or not the subunits are intrinsically active. An impressive collection of examples indicates that in vitro native quaternary protein structure is a prerequisite for enzymic activity (Jaenicke & Rudolph, 1977; Gerschitz et al., 1978; Groha et al., 1978; Yamato & Murachi, 1979). These conclusions are based on studies employing a variety of physicochemical techniques (Friedman & Beychok, 1979) and include kinetic evidence from experiments demonstrating that a rate-limiting bimolecular reaction occurs during the reactivation of denatured enzymes (Jaenicke, 1978). These studies include an analysis of the temporal relationship among reactivation, renaturation, and reassociation. On the

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