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Transition-State Structure for a Conformation Change of Ribonuclease

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The rate constants k_{12}^n for isomerization of the E_1H isomer ($pK_a > 8$ in H_2O) of ribonuclease-A to the E_2H isomer ($pK_a = 6.1$ in H_2O), determined from proton-uptake measurements by the temperature-jump technique, in mixtures of protium and deuterium oxides (atom fraction of deuterium n), are described by the equation $k_{12}^n = (733 \pm 16)(1 - n + [0.46 \pm 0.04]n)(1 - n + 0.69n)^2$ sec^{-1} at 25°C. On the basis of the absolute magnitude of the rate constant, the magnitude of the solvent isotope effect and the proton inventory, it appears that the rate-determining step is proton transfer to a water molecule from the imidazolium form of a histidine residue, with a product-like activated complex resembling a hydronium ion. The subsequent motion of the protein structure to generate the new isomer (conformation change) must then occur in a time approaching a vibrational period. Alternative but less likely mechanisms include rate-limiting protein reorganization concerted with proton transfer to water, rate-limiting diffusion of hydronium ion away from the enzyme, or "solvation catalysis" of protein reorganization.

INTRODUCTION

The astounding capacity of enzymes for accelerating chemical reactions is doubtless a resultant of many combined factors, but it seems certain that an important property for highly effective catalysis is the ability of the protein to exist in more than a single three-dimensional structure or conformation (*1*). Among other things, protein conformation changes may permit the enzyme to "shape itself" for optimal interaction with its ligands at the various stages of their conversion from reactants into products. Many conformation changes occur on the same time scale as events in the catalytic sequence, such as the formation and fission of covalent bonds, so that conformational reorganization of protein structure may become the rate-determining step in an enzyme-catalyzed reaction. The dynamics of protein conformation changes are thus of central importance for understanding the general origins of catalytic power of enzymes. The dynamic problem, in turn, raises the question of the transition state for conformation changes: Should this be regarded as encompassing a large volume of the protein structure or only a small functional unit of ordinary chemical dimensions? In other words, what is the structure of the transition state for a conformation change? Eventually, if such structures are determined and rationalized for a reasonable number of

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conformation changes, a predictive theory of the dynamics of conformation changes and of their connection with catalysis may emerge. In this paper, we are concerned with the structure of the transition state for an isomerization of the protein structure in the enzyme ribonuclease-A (*2*).

French and Hammes (*3*) some years ago studied a reversible thermal isomerization of ribonuclease-A near neutral pH, which may be important in preparing the enzyme for binding of substrate and catalytic function (*2, 4*). The rate of isomerization was strongly depressed in deuterium oxide, suggesting the role of proton transfer or hydrogen-bonding changes (*3*) in the rate-determining step, while the sigmoidal pH-rate profile with a pK of 6.1 in protium oxide led to the hypothesis that the less acidic (E_2H, p$K > 8$) form of the enzyme contained an aspartate–histidinium hydrogen bond, in or near the active site, which was broken to generate the more acidic (E_2H, pK 6.1) histidinium form of the enzyme (Eq. [1]).

$$E_1H \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} E_2H \overset{K_a}{\rightleftharpoons} E_2 + H^+ \qquad [1]$$

At that time, no definite structural assignment of the hydrogen-bonding amino-acid units was possible (*3, 5*). Another reasonable hypothesis (*5*) was that the His-48 "imidazole group is 'buried' in the 'acid stable' isomer of free ribonuclease . . . the pK value of the imidazole group . . . is considerably higher than normal. Upon 'exposure' (i.e., isomerization) the group assumes a pK value of about 6 for the free enzyme . . . ." An interesting light is thrown on this latter suggestion by a study of Roberts et al. (*6*) who observed line broadening of the C_2-H nmr resonance of His-48 apparently stemming from chemical exchange between the F_1H and E_2 conformational states. The exchange was catalyzed by acetate ion, which produced a relatively sharp resonance signal, but not by chloride ion. This suggested that acetate ion serves as a general-base catalyst in the deprotonation of His-48, which is coupled to the conformation change.

The large solvent isotope-effect (*3*), particularly in the light of the apparent general-base catalysis of the isomerization (*6*), seems to implicate proton transfer in the transition state for isomerization. However, solvent isotope-effects are ambiguous, since the observed rate change may result from a considerable effect of a single proton or from a combination of smaller effects of several protons[2] (*7*). Studies in mixtures of light and heavy water (*8*) ("proton inventories") allow one, in favorable cases, to list contributing protons and their isotope-effect contributions (*9*). From this list, it may be possible to

[2] A further consideration which arises in solvent isotope-effect studies of proteins is the concern that conformation may change in the deuterated solvent [W. P. Jencks, "Catalysis in Chemistry and Enzymology", pp. 276–278. McGraw-Hill, New York, 1969; J. J. Katz and H. L. Crespi in "Isotope Effects in Chemical Reactions", (C. J. Collins and N. S. Bowman, Eds.), pp. 319–320. Van Nostrand Reinhold, New York, 1970; J. F. Kirsch, *Annu. Rev. Biochem.* **42**, 205 (1973)]. It is important in this connection to realize that "an isotope effect is an isotope effect". There is nothing necessarily mysterious or incomprehensible about such conformation changes. They result merely from isotope effects on the conformational equilibrium constant and can be characterized in terms of the binding states of exchangeable protons in the various conformations concerned. In fact, even such remarkable phenomena as the very large rate and equilibrium solvent isotope effects on protein subunit association are analyzable and comprehensible with the aid of proton-inventory data [L. L. Houston, J. O'Dell, Y. C. Lee, and R. H. Himes *J. Mol. Biol.*, **87**, 141 (1974), on polymerization of microtubule protein, and J. A. K. Harmony and R. H. Himes, in preparation, on the more than 40-fold stabilization by deuterium oxide of the tetramer of formyl tetrahydrofolate synthetase].

TABLE 1

RECIPROCAL RELAXATION TIMES (METHOD A) FOR THE CONFORMATIONAL ISOMERIZATION OF RIBONUCLEASE-A IN BINARY MIXTURES OF PROTIUM AND DEUTERIUM OXIDES AS A FUNCTION OF pL[a] AT 25.0 ± 0.5°C

Atom fraction deuterium, n	pL[a]: Reciprocal relaxation time (sec^{-1})
0.000	7.63: 734, 755, 883, 677, 860, 651
	7.29: 876, 716, 771, 867, 836, 667
	7.00: 818, 966, 687, 693, 842, 892
	6.87: 747, 689, 849, 690, 817
	6.61: 978, 999, 813
	6.40: 809, 1045, 944, 1044, 964
	6.19: 1322, 1021, 863
	6.18: 1191, 847, 1009, 1423, 1190
	5.98: 1364, 1304, 1401
	5.78: 1444, 1574, 1713
0.249	7.73: 523, 533, 492, 581
	7.52: 488, 663, 589, 758, 587
	7.33: 478, 464, 618, 596, 390, 665, 452
	7.01: 649, 594, 532, 646
	6.85: 648, 718, 748, 937, 658, 862
	6.67: 837, 456, 1005, 869, 1355, 1212, 1134
	6.29: 1296, 1132
0.500	7.74: 282, 458, 260, 333, 448, 544
	7.17: 383, 402, 459, 408, 453
	6.90: 478, 486, 503, 433, 418, 520
	6.81: 331, 442, 717, 709, 395
	6.67: 480, 395, 417, 672, 574, 354, 607
	6.53: 583, 397, 618, 596, 510, 453, 654
	6.42: 498, 504, 505, 592
0.749	7.69: 283, 248, 266, 272, 249
	7.50: 262, 278, 304, 263, 223, 231
	7.31: 272, 321, 254, 291, 323, 271
	7.05: 268, 258, 226, 240, 313, 299
	6.79: 301, 375, 389, 265, 365, 326
	6.61: 369, 409, 320, 475, 336
	6.38: 602, 683, 371, 417, 386
	6.26: 500, 430, 453, 488
0.997	7.75: 146, 165, 228
	7.50: 245
	7.22: 263, 225, 173, 202, 191, 219
	7.20: 180, 181, 158, 172, 158, 214, 209
	6.92: 173, 226, 192, 236
	6.65: 192, 243, 305, 232, 249, 304, 303
	6.45: 331, 374, 353, 306, 303
	6.43: 192, 270

[a] pL is the negative logarithm of the activity of total lyonium species, determined as described in the text.

derive a structure, or partial structure, for the relevant transition state. Here we combine the results from such an investigation with other information to arrive at a transition-state structure for ribonuclease-A isomerization.

RESULTS

The experiments were carried out in two stages. In the first stage, relaxation times were determined at various pL values (= negative logarithm of total lyonium activity) in protium oxide and four binary mixtures of protium and deuterium oxides (atom fraction n of deuterium). The temperature-jump method was employed with photographic data acquisition and manual work-up (Method A), as described by French and Hammes (*3*). The reciprocal relaxation times are exhibited in Table 1. These were fitted to Eq. [2], corresponding to the scheme of Eq. [1] above, following French and Hammes (*3*), to obtain the rate and equilibrium constants of Table 2.

$$\tau_n^{-1} = k_{12}^n + \frac{k_{21}^n a_L}{K_a + a_L}. \qquad [2]$$

Since these data are insufficiently numerous and precise at $a_L \gg K_a$, usable values of K_a and k_{21} were not obtained. The data for k_{12}^n are good, however, and suggest a more extensive investigation of the k_{12} process at high pL, where fitting to Eq. [2] will not be required.

TABLE 2

RATE AND EQUILIBRIUM CONSTANTS IN THE CONFORMATIONAL ISOMERIZATION OF RIBONUCLEASE-A IN BINARY MIXTURES OF PROTIUM AND DEUTERIUM OXIDES AT 25.0 ± 0.5°C

Atom fraction deuterium, n	k_{12} (sec^{-1})	$10^{-2}k_{21}$ (sec^{-1})	$10^6 K_a$
0.000	739 ± 21	57 ± 69	9 ± 13
0.249	496 ± 39	160 ± 1400	10 ± 9
0.500	362 ± 26	2.6 ± 0.6	0.2 ± 0.1
0.749	243 ± 17	9 ± 11	1 ± 2
0.997	154 ± 29	3 ± 4	0.5 ± 1.4

This investigation constituted the second stage of experimentation. Automatic acquisition of the temperature-jump data with on-line computer work-up (Method B) was now used, permitting more extensive and precise measurements. All experiments were conducted at $a_L < K_a/30$ so that the variation in τ_n^{-1} (to within 3% or about half the experimental error) is equal to the variation in k_{12}^n. This rate constant is therefore obtained directly, and no statistical uncertainty connected with fitting to Eq. [2] attaches to its values. Data were obtained in protium oxide and 10 binary mixtures of protium and deuterium oxides, with 15–21 determinations at each solvent composition. The results are shown in Table 3.

Both data sets are plotted in Fig. 1. They are obviously totally congruent.

TABLE 3

RECIPROCAL RELAXATION TIMES (METHOD B) FOR THE CONFORMATIONAL ISOMERIZATION OF RIBONUCLEASE-A AT HIGH pL IN BINARY MIXTURES OF PROTIUM AND DEUTERIUM OXIDES AT 25.0 ± 0.5°C

Atom fraction deuterium, n	pL	$\overline{(1/\tau)}$ (sec^{-1})	Standard deviation	Range	Number of measurements
0.000	7.60	816	±40 (4.9%)	±76 (9.3%)	21
0.083	7.64	664	±49 (7.4%)	±90 (13.6%)	15
0.184	7.69	576	±34 (5.9%)	±64 (11.1%)	15
0.299	7.74	509	±42 (8.3%)	±79 (15.5%)	16
0.387	7.79	423	±37 (8.7%)	±74 (17.5%)	16
0.517	7.86	357	±24 (6.7%)	±51 (14.3%)	17
0.580	7.91	349	±26 (7.4%)	±49 (14.0%)	17
0.687	7.97	285	±22 (7.7%)	±45 (15.8%)	16
0.771	8.03	249	±20 (8.0%)	±37 (14.9%)	16
0.877	8.07	201	±10 (5.0%)	±22 (10.9%)	19
0.987	8.19	201	±12 (6.0%)	±20 (10.0%)	16

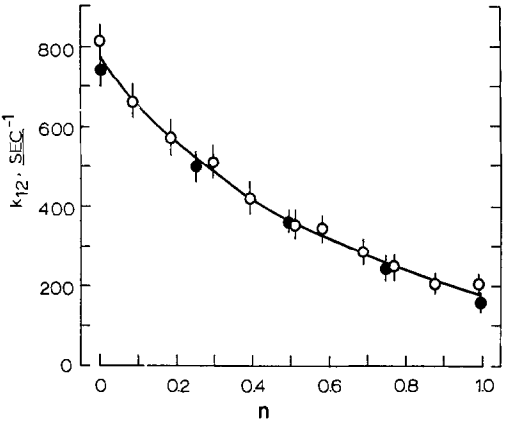


FIG. 1. Values of k_{12} (Eq. [1]) as a function of n, the atom fraction of deuterium in binary mixtures of protium oxide and deuterium oxide. The filled circles represent data obtained by Wang in 1972–1973 using Method A (Experimental Section), and the open circles are data of Rodgers and Gandour measured by Method B in 1974. The heavy line is a plot of the cubic equation, Eq. [6].

DISCUSSION

Proton Inventory

The net solvent isotope-effect of $k_{12}^0/k_{12}^1 = 4.7 \pm 0.4$ fully confirms the original observation of French and Hammes (*3*). Our more detailed picture of the mechanism will derive in part from the dependence of k_{12}^n on n, the proton inventory shown in Fig. 1. The variation of any rate constant k^u with n will be described (*8*) by Eq. [3],

$$k^n = k^0 \prod_i^v (1 - n + n\phi_i^T) / \prod_j^v (1 - n + n\phi_j^R), \qquad [3]$$

in which ϕ_i^T is the *isotopic fractionation factor* for the ith exchangeable hydrogenic site of the transition state and ϕ_j^R is the similar factor for the jth site of the reactant state.

Any isotopic fractionation factor $\phi_k \equiv \{[D]_k/[H]_k/[n/(1-n)]\}$ measures the deuterium preference of the kth exchangeable site relative to an average solvent hydrogenic site and is therefore the inverse isotope-effect (K_D/K_H) for conversion of a solvent hydrogenic site to the kth site. Thus a list of all the ϕ_i^T, obtained by fitting $k^n(n)$ with known or assumed ϕ_j^R, gives an inventory of transition state protons and the isotope effect associated with each one (*9*).

The first question therefore concerns the choice of the ϕ_j^R, the reactant-state ribonuclease fractionation factors.[3] The only ϕ_j^R which are necessary for Eq. [3] are those which change on conversion of reactants to transition state. Those which remain the same will contribute completely equivalent factors to both numerator and denominator and will therefore cancel. As will be shown below, the most reasonable chemical interpretation of the data reported here can be based on the assumption that all ϕ_j^R are either equal to unity or do not change on activation. Small departures from this assumption will affect the quantitative character of the proposals but will work no change in their qualitative form.[4]

Setting all ϕ_i^R in Eq. [3] equal to 1 yields Eq. [4], which shows that k_{12}^n can be represented as a polynomial in n (Eq. [5]) and that the order of the polynomial is in fact equal to the number of "active protons" in the transition state (i.e., protons which contribute to the solvent isotope-effect, and the binding of which is therefore altered on activation). The coefficients c_i of Eq [5] bear simple relationships to the θ_i^T and thus to the ϕ_i^T of Eq. [4].

$$k_{12}^n = k_{12}^0 \prod_i^{\nu} (1 - n + n\phi_i^T) = k_{12}^0 \prod_i^{\nu} (1 - n\theta_i^T); \qquad [4]$$

$$k_{12}^n = k_{12}^0 + c_1 n + c_2 n^2 + \ldots + c_\nu n^\nu. \qquad [5]$$

[3] The average fractionation factor for the exchangeable protons of a macromolecule is given by the "equilibrium isotope-effect" used in hydrogen exchange studies as a correction for converting experimental tritium or deuterium exchange data to the numbers of hydrogens (protiums) of various exchange rate classes. A review of exchange studies of ribonuclease is given by Richards and Wycoff (*2*). The "equilibrium isotope-effect" for exchange has been determined with varying degrees of precision for such species as synthetic polypeptides [for example, by W. H. Welch, Jr., and G. D. Fasman, *Biochemistry* **13**, 2455 (1974), and by S. W. Englander and A. Poulsen, *Biopolymers* **7**, 379 (1969)], ribonuclease [see, for example, S. W. Englander, *Biochemistry* **2**, 798 (1963)], human serum albumin (W. Lobunez and F. Karush, *J. Amer. Chem. Soc.* **81**, 795 (1959)], sperm-whale metmyoglobin [E. S. Benson, *C. R. Trav. Lab. Carlsberg* **31**, 235 (1959)], the hexapeptide ferrichrome [T. F. Emery, *Biochemistry* **6**, 3858 (1967)], insulin [A. Hvidt and K. Linderstrøm-Lang, *Biophys. Biochim. Acta* **18**, 308 (1955)] and nucleic acids [C. W. Lees and P. H. von Hippel, *Biochemistry* **7**, 2480 (1968); M. P. Printz and P. H. von Hippel, *Proc. Nat. Acad. Sci. U.S.A.*, **53**, 363 (1965); S. W. Englander and J. J. Englander, *Proc. Nat. Acad. Sci. USA* **53** 370, (1965)]. In all these investigations, either no discrimination was found or the heavier isotope was found to be favored by factors of up to about 1.2 (for tritium vs protium). This corresponds to a value of about 1.14 for deuterium vs protium. In any case, the methods used yield average values, while only the values for certain specific sites which change on activation are of importance for the proton inventory technique.

[4] A. J. Kresge, *J. Amer. Chem. Soc.* **95**, 3065 (1963), has shown that a number of small reactant fractionation factors for protons which are altered on activation may, under certain specialized conditions of combined experimental errors, lead to an apparent linearity in proton-inventory plots which actually have more than one active proton in the transition state. This kind of fortuitous effect is always an in-principle possibility and the final test of any conclusion based on proton-inventory data must therefore be agreement with a range of experimental or theoretical findings.

To determine how many protons are active in the transition state for ribonuclease-A isomerization we (a) subjected the data for $k_{12}^{n}(n)$ in Tables 2 and 3 to a series of least-squares fits,[5] to linear, quadratic, cubic, and higher-order polynomials in n; and (b) examined each term in these equations for statistical significance, to determine the order of the polynomial required by the data (*10*), and thereby the number of active protons in the transition state.

The best fit cubic polynomial $k_{12}^{n}(n)$ is that of Eq. [6], taking the 16 points of Fig.1 and Tables 2 and 3.

$$k_{12}^{n}(\text{sec}^{-1}) = 772 + (-1248 \pm 144)n + (1082 \pm 354)n^2 + (-440 \pm 234)n^3. \quad [6]$$

This function is plotted as the heavy line of Fig. 1. Statistical analysis by the *F*-test shows that the linear and quadratic terms in this equation are statistically significant at the 99.9% confidence limit. The cubic term is significant only at the 90% confidence limit. This demonstrates that $k_{12}^{n}(n)$ is nonlinear at the 99.9% confidence limit, thus requiring at least two active protons in the transition state, and that $k_{12}^{n}(n)$ requires a cubic term to the 90% confidence limit, suggesting a third active proton. Quartic and higher-order terms, in other fits, showed lower levels of significance.

The conclusion we draw is that not one but at least two and quite probably three protons must be invoked to account for the solvent isotope effect on ribonuclease isomerization.

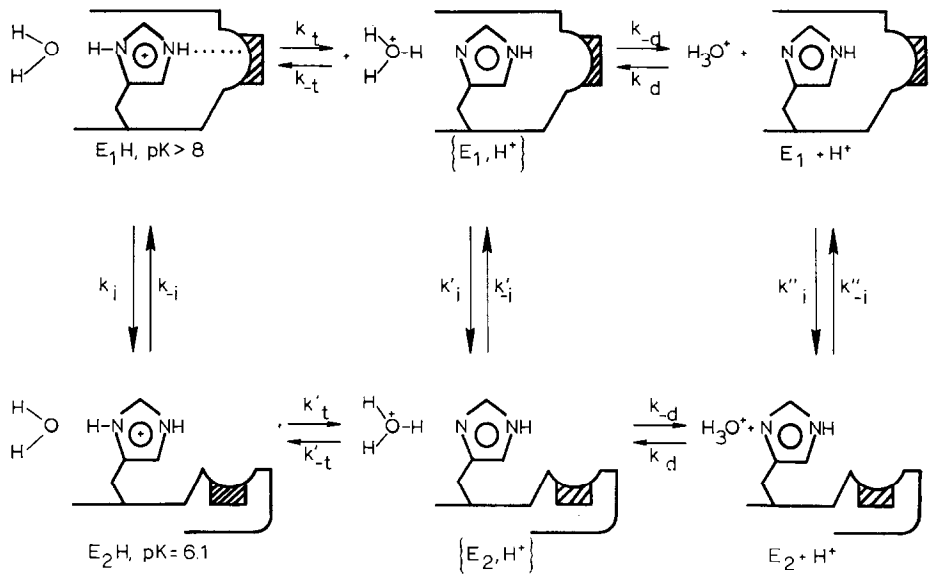
Since it is not certain that the "best-fit" coefficients of Eq. [6] are physically realistic, we decided to consider various candidate mechanisms and to compare the experimental data with proton-inventory equations based on these mechanisms. At this point, therefore, we repaired to the chemical level to try to design a two-proton or three-proton mechanism, preserving as much as possible of the well-founded mechanisms of previous workers (*3–6*). Two-proton schemes with realistic fractionation factors are relatively hard to construct, but the three-proton mechanism shown as Scheme 1 is attractive. In this scheme, the involvement of a water molecule in the isomerization process is envisioned in order to account for the "extra" protons required. Structural protons of the enzyme itself, except when generating primary isotope effects, cannot be expected to make the substantial isotope-effect contributions required here.[6]

Mechanism and Rate-Determining Step

In Scheme 1, the exchange of a histidinium residue between two environments is envisaged. In E_1H, at the top left, some characteristic of the local protein environment (symbolized by the three dots) is assumed to stabilize the imidazolium function, raising its pK above 8. In E_2H, at the bottom left, isomerization has altered the environment, removing the stabilizing interaction and producing a pK around 6. The steps along the

[5] The programs used were the polynomial regression (BMDO5R, version of 8/16/65) and nonlinear least-squares (BMDX85, rev. 5/14/69) programs of the Health Sciences Computing Facility, UCLA.

[6] The isotopic fractionation factors for hydrogens in hydroxyl, amino, and ammonium functions are all essentially equal to unity (*7*). Hydrogen bonding isotope effects for weak acid–weak base pairs tend to be very small, as exemplified by the fractionation factors for protein structural hydrogens (footnote 3) and formation of the HCl_2^- ion from HCl [J. Szydlowski, *Z. Naturforsch.* **30a**, 38 (1975)]. The solvent isotope effects on hydrophobic interactions are so small that their direction is a matter of controversy [D. J. T. Hill and C. Malar, *Aust. J. Chem.* **28**, 7 (1975); D. Oakenfull and D. E. Ferrwick, *Aust. J. Chem.* **28**, 715 (1975)].



SCHEME 1

top row of the scheme (left to right) represent the proton-switch and diffusion steps in the ionization of E_1H, while the bottom row portrays the same two steps in ionization of E_2H. Possible rate-determining steps involving three active protons[7] in the transition state are those for the two proton-switch steps (k_t and k_t'), the two isomerizations steps in which the hydronium ion is fully formed (k_i' and k_i'') and the two diffusion steps (k_d).

The isomerization steps (k_i' and k_i'') and the diffusion steps (k_d) are unlikely candidates for rate-limiting step because the overall solvent isotope effect is larger than would be expected for such steps. Since the hydronium ion has been fully formed in these steps, the solvent isotope effect should be essentially equal to that for ionization of a histidinium residue. This value (*11*) for both imidazolium and histidinium ions is only 3.6–3.7, while our overall effect is 4.7 ± 0.4. This suggests to us that the hydronium ion has not been preformed at the transition state, although the discrepancy between the expected and observed effects is small enough (a factor of 1.2–1.3) that it could conceivably arise from unknown sources.

We therefore reach the tentative conclusion that a proton-switch step (k_t or k_t') must be rate determining. The data of French and Hammes (*3*) permit us to choose between these two steps. We imagine a rapidly equilibrating mixture of $\{E_1, H^+\}$ and $\{E_2, H^+\}$ and inquire which is formed more rapidly from this mixture, E_1H or E_2H? The rate of formation of E_1H will be $k_{-t}[\{E_1, H^+\}]$ and the rate of formation of E_2H will be $k'_{-t}[\{E_2, H^+\}]$. We assume that k_{-t} and k'_{-t} will be nearly equal because they represent proton switches from a very strong acid (hydronium ion) to quite weak bases (pK's ~ 8.5 and 6.1, respectively). The rate constants for such highly exergonic processes should have a very small Brønsted β and be nearly independent of base pK. Then E_1H and E_2H will be formed with a relative rate equal to the ratio of concentrations $[\{E_1, H^+\}]/$

[7] The k_i step would yield no substantial isotope effect because the amine and ammonium fractionation factors are equal and hydrogen-bonding effects are small (see footnote 6).

$[\{E_2, H^+\}]$. If this ratio is the same as $[E_1]/[E_2]$ at equilibrium, then we can calculate it as follows: $[E_1]/[E_2] = [E_1H]K_a^{E1H}/[E_2H]K_a^{E2H} = (k_{21}/k_{12})(K_a^{E1H}/K_a^{E2H}) = (2468\ sec^{-1}/780\ sec^{-1})(10^{-8.5}/10^{-6}) \simeq 1/100$. Thus E_2H should be formed from the equilibrating mixture of $\{E_1, H^+\}$ and $\{E_2, H^+\}$ about 100-fold faster than E_1H. Thus the rate-determining step in the isomerization should be the proton switch from E_1H to water.

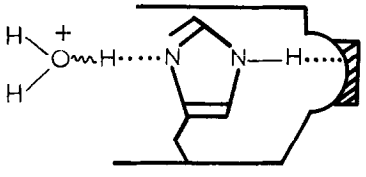
In addition we can estimate the rate constant to be expected for conversion of E_1H to $\{E_1, H^+\}$ from Eigen's kinetic model (*12*) for weak-acid ionization in aqueous solution (Eq. [7] corresponding to the top row of Scheme 1). According to Eigen, the rate-determining step in this sequence is diffusion apart of E_1 and H^+ (k_{-d} step).

$$E_1H \underset{k_{-t}}{\overset{k_t}{\rightleftharpoons}} \{E_1, H^+\} \underset{k_d}{\overset{k_{-d}}{\rightleftharpoons}} E_1 + H^+. \qquad (7)$$

The overall equilibrium constant is just $K_a^{E1H} \sim 10^{-8.5}$. From Eq. [7], $K_a^{E1H} = (k_t/k_{-t})(k_{-d}/k_d)$. Then $k_t = K_a^{E1H}k_d(k_{-t}/k_{-d})$. For diffusion to be rate-determining, $k_{-t}/k_{-d} \simeq 10$ or more. We also know that $k_d \sim 10^{10}\ M^{-1}\ sec^{-1}$. Thus $k_t \sim 10^{8.5} \times 10^{10} \times 10 \sim 10^{2.5} = 300\ sec^{-1}$. This very rough estimate compares extremely well with the experimental value of k_{12} between 700 and 800 sec^{-1}. Thus the experimental value of k_{12} is essentially equal to k_t, the value expected for proton transfer from an acid of pK 8.5 to a water molecule.

Transition-State Structure

The arguments given above lead to structure **1** as the most likely representation of the transition state for the ribonuclease conformation change. As indicated in the last paragraph, a proton switch between two bases of very different basicity should lead to a transition state resembling the stronger acid. Therefore in **1** we show the charge on oxygen as essentially unity.



1

The two "outer" protons of the hydronium moiety should have isotopic fractionation factors about equal to those of the free hydronium ion (0.69), while the bridging proton ("in flight" in the transition state) should generate a larger normal isotope effect (thus a smaller fractionation factor). These postulations allow us to rewrite Eq. [3] in the form of Eq. [8] where ϕ^* is the fractionation factor of the bridging proton. Further transformation to Eq. [9] shows that a test of the hypothesis is possible; a plot of the left-hand side of Eq. [9] vs n should, if **1** is correct, be linear. Such a plot is given in Fig. 2 and is satisfactorily linear. Its slope and intercept yield $k_{12}^0 = 733 \pm 16\ sec^{-1}$ and $\phi^* = 0.46 \pm 0.04$. This corresponds to an isotope effect $k_H/k_D = 2.2 \pm 0.2$ for the bridging proton of **1**. Just such a small, normal isotope effect is what is expected for a very asymmetric proton-switch transition state.

$$k_{12}^n = k_{12}^0(1 - n + n[0.69])^2(1 - n + n\phi^*); \qquad [8]$$

$$k_{12}^n/(1 - n + n[0.69])^2 = k_{12}^0(1 - n + n\phi^*). \qquad [9]$$

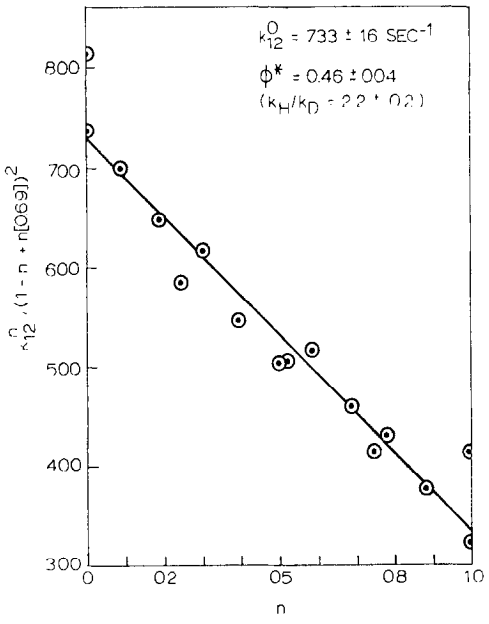


FIG. 2. A plot of the left-hand side of Eq. [9] versus n, the atom fraction of deuterium. Equation [9] holds that this plot should be linear. Its slope and intercept define ϕ^* and k_{12}^0, which are shown at the upper right.

Implications of the Mechanism

Figure 3 shows a free-energy diagram that describes the system on the basis of the mechanistic model. The mechanism shown in Fig. 3 implies that the protein reorganization step (HAR) is exceedingly fast. A semiquantitative estimate of its rate constant can be obtained as follows. If the isomerization step indeed occurs rapidly subsequent to a rate-determining proton transfer, with the hydronium ion of $\{E_1, H^+\}$ a trapped "spectator" at the protein reorganization, then the isomerization (k_i') must be, say, tenfold faster than the reverse proton transfer (k_{-t}). This in turn is about tenfold faster than escape of hydronium ion from the vicinity of $E_1(k_{-d})$ (*12*). The protein reorganization is thus a hundredfold faster than the diffusion of hydronium ion away from the ionized enzyme. Since $\{E_1, H^+\}$ probably constitutes no more than 1% of the ionized E_1-species (that is, very few molecules will be existing in the solvent-caged form), k_d/k_{-d} (the equilibrium constant for combination of E_1 and H^+ to generate $\{E_1, H^+\}$ must be no greater than 10^{-2} M^{-1}. If $k_d \sim 10^{10}$ M^{-1} sec^{-1}, k_{-d} is then about 10^{12} sec^{-1}, which puts k_i' near 10^{14} sec^{-1}. Thus the characteristic time for the isomerization step itself, on this model, 10^{-14} sec, approximates the period of a molecular vibration of frequency 3300 cm^{-1}. Such a rapid motion of more than a very small segment of protein structure seems unlikely and indeed, the rapidity would be astonishing for any motion of heavy atoms. It seems quite probable, therefore, that we are dealing with a situation of the sort recently elucidated by Choi and Thornton (*13*) who found cases in which a rate-determining process led not to a stable structure but directly into the activated complex for a second process. In the isomerization of ribonuclease-A, the transfer of

the histidinium proton to adjacent water, resulting in loss of a stabilizing interaction between histidinium and other elements of the protein structure, may "liberate" the protein structure at a point of maximum energy, where restoring forces are absent and the reorganization (HAR in Fig. 3) may proceed with no energy requirement whatever.

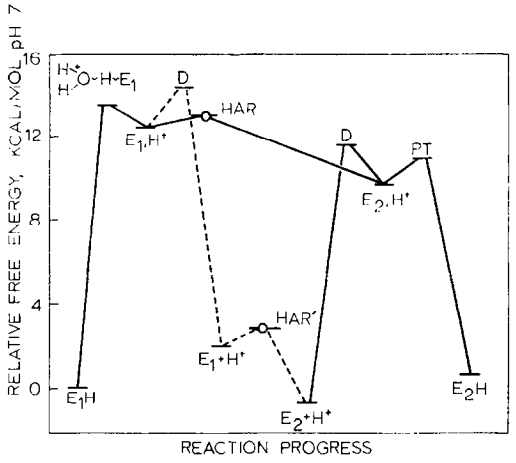


FIG. 3. A plot of relative free energy along the reaction pathway from E_1H to E_2H, formulated according to the mechanism of Scheme 1 with the restrictions discussed in the text. The solvent-caged species $\{E_1, H^+\}$ is formed from E_1H in the rate-determining proton-transfer step. The protein isomerization step (HAR = heavy-atom reorganization) is relatively fast, while diffusion (D) of H^+ away from E_1 is relatively slow. After HAR generates $\{E_2, H^+\}$, a rapid proton transfer (PT) and slow diffusion (D) distribute the material between E_2H and $E_2 + H^+$. Isomerization through HAR′ (a transition state of lower free energy than HAR) in the absence of H^+ is blocked off by the slow diffusion step.

Therefore both processes, HAR and HAR′, represented as having very small barriers in Fig. 3, may have no barriers whatever. It is interesting that Cathou et al. (*14*) have shown the isomerization to involve no change in circular-dichroism or ultraviolet spectra for the enzyme. This is consistent with a quite small structural change and, thus, with the absence of a substantial barrier to reorganization.

Alternative Mechanisms

In addition to the further possibility mentioned above, that the isomerization or diffusional steps are in fact rate-limiting, with solvent/isotope-effect discrepancies to be explained away by invocation of effects of unknown origin, a more interesting alternative exists. This is a combined proton-transfer and protein reorganization process. In one form of this mechanism, the reaction coordinate consists of both proton motion and protein reorganization so that E_1H is carried directly through a *single* transition state over to $\{E_2, H^+\}$ (by a route not depicted in Scheme 1). In so far as the proton-inventory probe goes, the transition state for such a reaction should be indistinguishable from **1**. A different form of this mechanism holds that tight, transition-state hydrogen bridging from the hydronium ion to the enzyme facilitates the reorganization process ("solvation catalysis (*7*, *15*)"). Again, the transition state near the hydronium center would be the same as in **1**. Both of these proposals seem

less likely to us than the simple proton-transfer transition state proposed above because (i) if these proposals were correct, the magnitude of k_{12} would agree with the rate constant predicted from the Eigen model only by coincidence, (ii) it is difficult to see how the protein motion could be mechanically coupled to the proton transfer to produce the "concerted" mechanism, and (iii) it is difficult to see how the protein motion could readily be accelerated by the solvation–catalysis interaction.

Comparison with Previous Work

The mechanism of Scheme 1 (or any concerted variant) requires (*7*) that the reaction show no equilibrium solvent isotope-effect ($K_{12}^{H}/K_{12}^{D} = 1.0$, where $K_{12} = k_{12}/k_{21}$) and a "normal" solvent isotope effect on $K_a(pK_a^D - pK_a^H \sim 0.6)$. In French and Hammes's report (*3*), their data were so fit to Eq. [2] that values of $K_{12}^{H}/K_{12}^{D} = 0.7$ and $\Delta pK_a \sim 0.8$ were generated. Examination of their plots suggested, however, that no material loss in goodness of fit might result if values were chosen which were in accord with Scheme 1. Indeed when the assumptions that both k_{12}^{H}/k_{12}^{D} and $k_{21}^{H}/k_{21}^{D} = 4.77$ and that $pK_a^D = 6.7$ were used to generate new fitting parameters for the French–Hammes data in D_2O, a ratio of the rms deviation to rms data point of 12.3% was obtained, while the original parameters produce 10.4%. This minor difference confirms that the original data are sufficiently in accord with Scheme 1 not to militate against its acceptance.

Conclusion

In this isomerization of ribonuclease-A, the absolute magnitude of the rate constant, the overall solvent isotope effect, the proton inventory, and a previous observation (*6*) of general-base catalysis combine to suggest a transition-state structure resembling a histidine–hydronium ion assembly with a proton "in flight" from the histidine toward a water molecule, located very close to the latter. Thus the transition-state structure is that for a simple chemical event, a proton transfer, and is highly localized within the protein structure. The actual motion of the polypeptide chain must then occur very rapidly and subsequently, possibly without any energy barrier. While other possibilities remain, this seems the most plausible view of the mechanism of the conformation change.

EXPERIMENTAL

Materials

Bovine pancreatic ribonuclease-A was obtained as a phosphate-free lyophilized powder from Worthington Biochemical Corp. and was used without further purification. Protium oxide was deionized through an ion-exchange column and double distilled and was boiled before it was used. Deuterium oxide was obtained from Diaprep Inc. and Stohler Isotopes and was boiled under nitrogen before use. The deuterium content was checked by nmr with acetonitrile as standard. Phenol red and chlorphenol red were reagent-grade crystalline materials from Fisher Scientific Co., and they were used without further purification. Potassium nitrate was an analyzed reagent-grade powder from Baker Chemical Co.

Preparation of Solutions

For the experiments in pure H_2O or D_2O, crystalline ribonuclease-A was dissolved in 10 ml of water solution which contained 0.1 *M* potassium nitrate and 0.02 m*M* of the indicator (phenol red or chlorphenol red). The concentration of the enzyme was 0.1 m*M* in most cases. For the experiments in H_2O–D_2O mixtures, a known volume of H_2O solution containing 0.1 *M* potassium nitrate and 0.02 m*M* indicator was weighed, and it was then mixed with a weighed, known volume of D_2O solution containing 0.1 *M* potassium nitrate and 0.02 m*M* indicator. For experiments using Method A (below), the weight of potassium nitrate was then subtracted from each water solution and thus the deuterium atom fraction of the H_2O–D_2O solution could be calculated. In the calculation, the weight of the indicator added was neglected, since the amount was quite small. Crystalline ribonuclease-A was then dissolved in 10 ml of this solution. The concentration of the enzyme is also near 0.1 m*M*. For experiments using Method B (below), the solutions were collected at the end of the experiment. A 1-ml sample was weighed. An appropriate, weighed amount of purified acetonitrile was added so that the integration ratios of nmr peaks for the methyl group of acetonitrile and the solvent protons were approximately equal. Twelve sweeps of the signals were used to determine the integrated intensities. The value of *n* was calculated from the relative integrated intensities of the HOL signal and the CH_3CN signal and the known weights of soluti on potassium nitrate, enzyme, and indicator.

pL Measurements

In pH measurements in H_2O solution, 0.1 *N* sodium hydroxide solution or 0.1 *N* hydrochloric acid solution (about 10–20 μl) was injected to adjust the pH value to the desired value. Measurement of pH values was performed with a Radiometer pH meter with an expanded scale and a combination electrode (No. GK 2302C). In pL measurements in D_2O solution, sodium hydroxide solutions in D_2O and hydrochloric acid solution in D_2O were used to adjust the pL to the desired value. In H_2O–D_2O solution, sodium hydroxide solutions in the H_2O–D_2O mixture with the same deuterium fraction as that in enzyme solution were used to adjust the pL value. Once again, the amount of the titrant added was so small, the effect on both the deuterium fraction in the solution and the concentration of potassium nitrate in the solution could be neglected. For pL determinations in mixtures, we used the relationship between the pH meter reading and the real pL value, established by Salomaa, Schaleger, and Long (*16*),

$$\text{pL} = \text{pH meter reading} + \Delta\text{pH},$$

$$\Delta\text{pH} = 0.331 + 0.0766 \times n^2,$$

or an equivalent relation of ours (*17*),

$$\text{pH} = (0.2208 \pm 0.0122) + (0.1732 \pm 0.0130)n^2$$

Kinetic Measurements (Method A)

All the temperature-jump experiments were performed with a Durrum stopped-flow, temperature-jump spectrophotometer (Model 100 and 150). The temperature of the

enzyme solution was jumped from 17 to 25°C. The decrease in the transmittance of the solution due to the increase of the ionized form of the indicator was then monitored by an oscilloscope. The oscilloscope trace was photographed with a Polaroid camera. The "best" trace was drawn and read by a micrometer. The first-order rate constant was calculated by a nonlinear least-squares computer program. In the pH region where phenol red was used as an indicator (pH 6.8–7.8), the absorption change of the indicator after the temperature jump was measured at 560 nm; in the chlorphenol red region (pH 5.7–6.8), the absorption change was measured at 580 nm.

Kinetic Measurements (Method B)

The data from the photomultiplier of the Durrum spectrophotometer were collected by a Biomation 802 transient recorder, interfaced to a Hewlett–Packard 2100A computer and a Textronix 604 oscilloscope. Data were collected for a period of $\sim 10\tau$ and displayed on the oscilloscope for visual examination, in order to detect errors from such sources as unusual cooling or cavitation. For acceptable runs, the rate constant $(1/\tau)$ was evaluated from data for a period of approximately 5τ by a nonlinear least-squares fit to a first-order exponential curve. Standard deviations in $1/\tau$ were typically 3% within a given run. For each solvent composition, at least 16 rate constants were evaluated. Those more than 2 SD from the mean were rejected.

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