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Oxidized RNase as a Protein Model Having No Contribution to the Hydrogen Exchange Rate from Conformational Restrictions*

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Abstract. As oxidized RNase is a model for the random conformation state of RNase, the hydrogen exchange kinetics of oxidized RNase approximate the intrinsic conformation-independent chemical exchange rate of the native protein. The energy of activation, the pH_{min}, and the k_{min} of oxidized RNase exchange rates are similar to those reported for amino acid homopolymers. However, unlike the exchange from homopolymers, the exchange from oxidized RNase is characterized by a distribution of first-order rates. This distribution is important to the analysis of exchange from native proteins in terms of classes of sites which share common structural properties.

The primary goal of protein hydrogen exchange studies is to probe the dynamics of protein conformation. Toward this end it is necessary to estimate the intrinsic conformation-independent chemical exchange rate which may then be used to analyze the overall kinetics of native protein hydrogen exchange for contributions from factors reflecting protein conformation. Synthetic homopolyamino acids are often used as models to estimate the chemical exchange rate.[1–3] In all reported cases the out-exchange of hydrogens from homopolymers with half-lives in excess of 2.5 min can be described by a single first-order rate constant, indicating the chemical homogeneity of the exchanging sites. However, since a protein is a heteropolymer in which exchanging amide hydrogens may experience varied local environments, it is reasonable to question whether the exchange from an unfolded protein in the so-called random state also follows first-order kinetics, especially in view of side-chain-dependent rate differences reported for small peptides.[4,5] The problem is of importance to all hydrogen-exchange studies, since analysis of exchange curves as sums of independent linear terms having conformational implications requires that the basic exchange rate constants of all individual sites in a model free of conformational restrictions show no variation and thus that the variations in apparent exchange rates are only produced by protein conformational factors.

A suitable model compound is a polypeptide which has little or no nonrandom folding but which does have the same primary sequence as the protein under consideration. For RNase such a model compound is readily available in performic acid-oxidized RNase, and, accordingly, we have studied the exchange kinetics of oxidized RNase. We find that although the value of the exchange rate is comparable to that reported for homopolyamino acids, the exchange does not follow

first-order kinetics. Therefore, if each site exchanges with a single first-order rate constant, the curved first-order plots of exchange from oxidized RNase represent a distribution of first-order rates.

Materials and Methods. Performic acid-oxidized RNase prepared[6] from bovine pancreatic RNase (Sigma Co., type III-A) gives a single band in polyacrylamide gel electrophoresis, pH 8.6 and 7.5% gel, which is clearly different from that of the native RNase. The circular dichroic spectrum of oxidized RNase, as recorded on a Cary 60 recording spectropolarimeter with circular dichroism attachment, is identical to that reported for oxidized RNase[7] and for reduced RNase.[8] It is dominated by a large 198-nm trough indicating a predominant random-coil structure although a formal α-helix content of approximately 5% is indicated.[7,8]

Guanidinated-oxidized RNase was prepared by treating the oxidized RNase with *o*-methyl isourea.[9]

The exchange method is a modification[10] of the technique introduced by Englander.[11] Oxidized RNase was tritiated under conditions (pH 6, 60–62°C for 15 min) of complete in-exchange for the native RNase.[12] Out-exchange solutions are buffered by 0.04 M glycine-HCl at the pH indicated.

Out-exchange in the presence of guanidine-HCl (Heico, ultrahigh purity) or sodium dodecyl sulfate is initiated by removing the excess tritium on a Sephadex column equilibrated with additive at the appropriate pH. The experiments with urea (deionized, recrystallized) and trifluoroacetic acid are different in that excess label is removed on a buffer equilibrated column; after the peak fractions are pooled, the urea or trifluoroacetic acid is added at the appropriate pH.

Calculations. The number of hydrogens/molecule unexchanged (H_{rem}) was calculated as before,[10] using a molar extinction of 8.40×10^{-6}/cm at 275 nm.[13]

The effects of 6 M guanidine-HCl on the chemical exchange rate constant have been estimated in the following way. There is a decrease in water concentration in 6 M guanidine-HCl to 31.7 M compared to 55 M for pure water. The water activity in 6 M guanidine-HCl at 25°C is calculated[14,15] to be 0.78. Since the self-dissociation constant for water is independent of salt concentration, and since we hold the hydrogen ion activity constant in both solutions, the drop in water activity with added 6 M guanidine-HCl will result in a concomitant decrease in the hydroxyl ion activity by about 25%.

A decrease in the hydroxyl ion activity will affect the rate constant of an individual site which can be expressed as

$$k = k_0 + k_H(H^+) + k_{OH}(OH^-), \quad (1)$$

where k_0 is the rate constant for the spontaneous reaction, k_H that for the acid-catalyzed reaction, and k_{OH} that for the base-catalyzed reaction.[4] Although k_0 will decrease as the molarity of water decreases in 6 M guanidine-HCl, the absolute value of this constant is so small that a further decrease in it would not influence the overall rate.[4] Thus, provided no other factors are present, the term for the base-catalyzed reaction, k_{OH}, will be the only one affected in Eq. (1) when we go from a dilute buffer solution to a 6 M guanidine-HCl at the same pH. If we assume the mechanism proposed by Berger *et al.*,[16] the rate-determining step reflected in k_H and k_{OH} is dissociation from the amide group. However, precise calculations of the effects of a drop in hydroxyl ion activity are complicated by the possibility of preferential binding of guanidine-HCl to oxidized RNase although native RNase does not bind the salt preferentially.[15] In addition, the contributions of hydroxyl ion catalysis to the total rate varies with the position of the pH_{min}, which could be changed if there were specific binding of the salt to oxidized RNase.

In summary, hydrogen exchange in 6 M guanidine-HCl is expected to show a decreased rate compared to that in dilute buffer due to decreased hydroxyl ion activity in 6 M guanidine-HCl. The magnitude of the decrease is difficult to calculate because of uncertainties in several factors, but a reasonable estimate is 20% ± 10%.

Results and Discussion. In the interpretation of hydrogen exchange kinetics of native RNase we employ oxidized RNase as a model having no contributions to the exchange rate from protein conformation.[12] Although the unfolded states of globular proteins may be different from the idealized random coil defined in polymer chemistry,[17] the extent to which hydrogen exchange mirrors the presence of residual structure is not clear. However, the unfolded protein, not the idealized random coil polypeptide, is the pertinent model for hydrogen exchange experiments with native proteins.

We find that, unlike homopolymers,[1-4] oxidized RNase exchange of the peptide amide hydrogens does not follow first-order kinetics as has been reported.[1] Figure 1 shows a semilogarithmic plot of the out-exchange from the oxidized

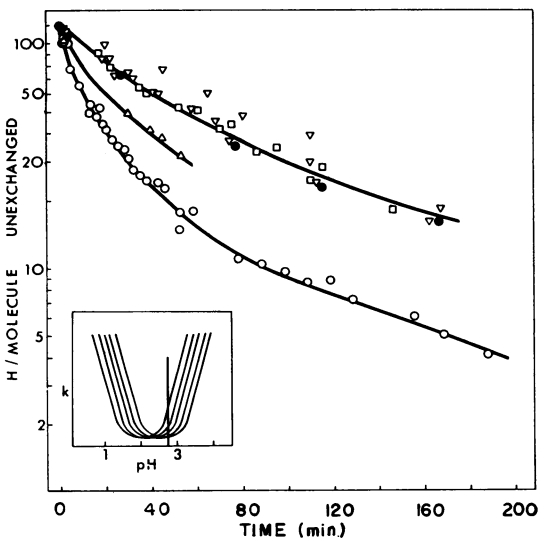

FIG. 1.—The kinetics of out-exchange from fully tritiated oxidized RNase at 2°C; at pH 3.15, ○; at pH 2.8, △; at pH 2.5, ▽; at pH 2.3, ●; at pH 2.0, □.

Inset: The diagram illustrates the distribution of first-order rate constants which would be observed if at a single pH one measured simultaneously the exchange rates of five exchangeable sites having different pH_{min}'s but identical k_{min}'s. See text for full explanation.

RNase at various pH's and 2°C. A pH-dependent rate minimum is characteristic for hydrogen exchange of amide hydrogens, since the chemical exchange is both acid- and base-catalyzed, and we follow the notation of Leichtling and Klotz[4] by denoting the pH and the rate constant at that point by pH_{min} and k_{min}. The value of pH_{min} depends upon the polymer under study, but it is usually between pH 2 and 3. At no pH can the exchange from oxidized RNase be described by a single first-order rate constant. If exchange from a single site occurs by first-order kinetics, and there is abundant evidence from model compounds that it does,[1,18] then the curvature of the plots in Figure 1 must be caused by a distribution of first-order rate constants. This has many important ramifications for the interpretation of hydrogen exchange data from native proteins, but we must consider the structure of oxidized RNase before drawing conclusions as to the factors causing the apparent distribution of rates.

In a series of studies emanating from the Carlsberg Laboratories,[13,19-21] it was shown that the cleavage of the four disulfide bonds drastically alters the native globular configuration of RNase to an extended, unfolded molecule having little

or no regular remaining structure. The question of whether oxidized RNase has any nonrandom structure has not been unequivocally resolved. Although the dominant form of oxidized RNase is clearly a random coil, residual structure due to localized or overall restrictions on the radius of the coil cannot be definitely excluded.[19-24] Therefore it might be argued that the wide range of first-order exchange rates of oxidized RNase is due to the residual structure rather than to the inherent distribution of rates for the various exchanging sites. We have addressed this question by testing the effect of strong denaturants on the oxidized RNase exchange rate. Such experiments with 6 M guanidine-HCl are shown in Figure 2.

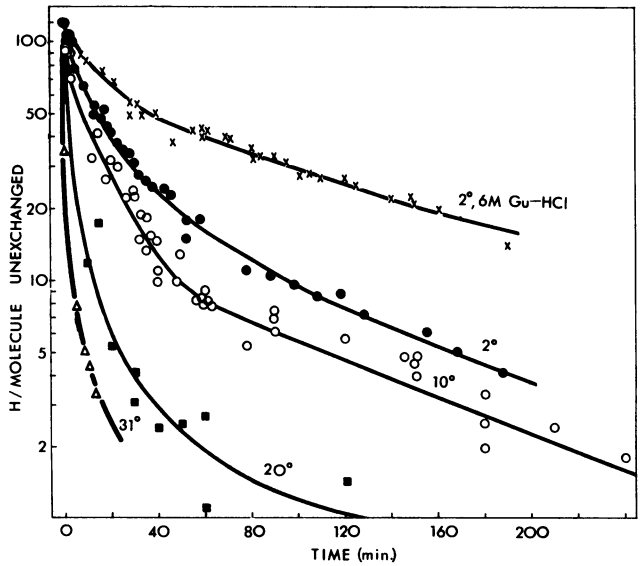

FIG. 2.—The kinetics of out-exchange from fully tritiated oxidized RNase at pH 3.15; at 2°C, ●; at 10°C, ○; at 20°C ■; at 31°C, △. The solid curve at 2°C is calculated from Eq. (1); see text for details. The kinetics of out-exchange from the fully tritiated protein at pH 3.15, 2°C, with 6 M guanidine-HCl added to the out-exchange solution, X.

When 6 M guanidine-HCl is added to the oxidized RNase exchanging medium, the exchange rate at 2°C (Fig. 2) and at 10°C (unpublished data) is not increased as would be expected[25] if the salt destroyed some residual structure, thereby making available for exchange sites formerly constrained. At 2°C and at 10°C the rate of exchange in 6 M guanidine-HCl compared to that in dilute buffer is decreased by about 8–10% but it retains the same marked curvature of the first-order plot. The exchange in 6 M guanidine-HCl shows a distribution of rates similar to that in dilute buffer, and the change in value of the rates is very close to that estimated from calculations of the effect of 6 M guanidine-HCl on the hydroxyl ion concentration.

Thus, although hydrogen exchange is not necessarily sensitive to all rotational restrictions and side-chain interactions, the destruction of some structural

rigidity by 6 M guanidine-HCl, as indicated by fluorescence polarization studies,[24] is not detected in the exchange properties. It is nevertheless possible that oxidized RNase has some degree of structure which is not susceptible to disruption even by 6 M guanidine-HCl,[17] leaving an argument that the curvature of the first-order plots may be due to residual structure that is not disrupted by 6 M guanidine-HCl. However, even if this explanation were true, oxidized RNase still is the best compound, since it is the most random molecule available with the same primary sequence as native RNase and any unfolded state of native RNase would certainly be as restricted. In addition, it is not necessary to invoke residual structure of unknown character in oxidized RNase to explain the curvature of the first-order plots and its variation. It may be viewed as a consequence of differences in pH_{min} and k_{min} for each peptide amide site. This is the interpretation favored by us, and our arguments for it are the following.

The detailed out-exchange curves in Figure 1 extend over four half-lives. The most extensive studies were conducted at pH 3.15 because the best available data on the thermal unfolding were obtained at that pH.[26] The exchange at pH 3.15 and 2°C (Fig. 2) of the 119 peptide amide hydrogens can be described with sufficient precision by four first-order terms:

$$H = 20e^{-0.345t} + 48e^{-0.0862t} + 30e^{-0.0345t} + 21e^{-0.00862t} \quad (2)$$

H is the total number of hydrogens unexchanged and the time, t, is in minutes. This equation contains the minimum number of terms necessary[27] although the terms themselves are not considered to represent the actual exchange rate of any site or group of sites. The drawn curve for pH 3.15, 2°C, in Figure 2 is the calculated curve generated by Eq. (2). The agreement with experimental points is not surprising, since within the experimental precision attainable, most exchange curves can be represented by four independent terms.[18] The distribution of rates in Eq. (2) is 40-fold, considerably greater than the difference between k_{min} for different dipeptides.[3-5] We attribute this large variation to differences in local environment of the exchanging sites.[28] That is, in a heteropolymer each amide nitrogen has a different local environment due both to inductive effects of the side-chain groups and also to solvent effects of the local milieu. Therefore, each amide nitrogen may be a unique chemical entity and as such may have a unique k_{min} and pH_{min}. Thus, even if all the k_{min}'s are the same for each site, when the pH_{min}'s are not the same, the exchange at a given pH will be at a different rate for each site. This may be visualized in the diagram (*inset*) of Figure 1. The diagram shows the curves we could get if the pH dependence of, for example, five sites were measured separately; the curves are similar in shape but each has a minimum at a different pH. If at a single pH, e.g. pH 2.6, we then measure the exchange of all five sites simultaneously—the situation we encounter in a protein—we would observe a distribution of k's with values equivalent to the points at which the vertical line intersects the curves. If in addition to having varying pH_{min}'s the sites also have varying k_{min}'s, then the distribution could be even greater.

The k_{min} and pH_{min} for tritium exchange from poly-*dl*-alanine and poly-*dl*-lysine have been reported by Englander and Poulsen.[3] Since the poly-*dl*-

alanine data are the more complete, we chose them for comparison with data from oxidized RNase in Table 1. It can be seen in Table 1 that the major part of the rate interval for oxidized RNase is faster than that of the poly-*dl*-alanine. An explanation is that the k_{min} and/or pH_{min} of the faster exchanging sites is

TABLE 1. *The exchange rate at and near the pH_{min} for oxidized RNase and poly-dl-alanine.*

	Oxidized RNase at 2°C*	Poly-*dl*-alanine at 0°C†
pH_{min}	pH 2.5	pH 2.9
k at pH_{min}	$7.3\text{–}27.5 \times 10^{-3}min^{-1}$	$8.4 \times 10^{-3}min^{-1}$
$pH_{min} + 0.6$	pH 3.1	pH 3.5
k at $pH_{min} + 0.6$	$0.86\text{–}34.5 \times 10^{-2}min^{-1}$	$1.32 \times 10^{-2}min^{-1}$

* The oxidized RNase rate constant, k, is expressed as a distribution of first-order rate constants fitting the observed exchange curve. Thus the pH of minimum rate, pH_{min}, is the pH at which the mean average of the distribution has the lowest value of first-order rate constant.

† Data from Englander and Poulsen.[3]

different from that of the slower ones. Support for this is the pH study shown in Figure 1. If the observed exchange curve is, for example, a composite of four first-order lines representing specific groups of sites and if the rates of the groups have an identical pH_{min}, then the observed exchange curves would be shifted by a common interval for each increment of pH. That is, if dk/dpH is the same in all rate groups, all rate constants should be changed by a common multiplier and consequently the distribution of rate constants should remain unchanged. However, an inspection of Figure 1 shows that the distribution of rates is lowered from pH 3.15 to pH 2.5. The exchange curve at pH 2.5 can be represented by a smaller number of independent first-order terms:

$$H = 62e^{-0.00726t} + 57e^{-0.0275t} \tag{3}$$

and Eq. (3) gives a satisfactory fit to the pH 2.5 data in Figure 1. The fastest exchanging sites are more affected than the slow ones. The slower group is evidently near its minimum at pH 3.15, whereas the minimum for the faster group is shifted towards lower pH.

We have also studied the effects of the exchange rate at pH 3.15 by 3 M urea, 5 and 10% trifluoroacetic acid, and 0.025 M sodium dodecyl sulfate. Like 6 M guanidine-HCl, 3 M urea has no effect on the rate. The 5 and 10% trifluoroacetic acid decreases the rate by approximately 5% and 0.025 M sodium dodecyl sulfate increases it by approximately 10%. Taken alone, the trifluoroacetic acid and sodium dodecyl sulfate results cannot be interpreted unequivocally, since the effect of these additives could be due either to a change in the structure of the molecule or to a change of k_{min} or pH_{min} for some or all sites. In view of the work of Klotz and his collegues[28] the latter interpretation is the more likely.

It has been reported that guanidinium hydrogens may exchange slower than peptide hydrogens.[29] Since in Figure 2 the last 12 $\pm$ $2H_{rem}$ of the 10° curve appear to lie in a straight line on the semilogarithmic plot, it may be argued that these hydrogens are bound to sites other than peptide amide nitrogen. Therefore the effect of guanidination on the exchange from oxidized RNase was tested. If the last hydrogens are bound to guanidinium nitrogen, then the number of H_{rem} in the last straight line portion of the curve would greatly increase. How-

ever, guanidination does not change the rate of exchange from oxidized RNase at pH 3.15, 2°C.

The temperature dependence of the out-exchange from oxidized RNase at pH 3.15 is shown in Figure 2. The apparent energy of activation, calculated as described earlier,[10,30] is $E_{ap}^* = 15$–18 kcal, in good agreement with published values for polymers.[1-4]

In summary we conclude: (1) Oxidized RNase is an appropriate model for protein hydrogen exchange in which there is no contribution to the rate from secondary or tertiary structure. Although some optical properties of oxidized RNase indicate possible residual structure, there is no further loss of structure by the addition of 6 M guanidine-HCl detectable by hydrogen exchange studies. (2) The exchange from oxidized RNase is characterized by a distribution of rates. (3) The energy of activation, the pH_{min}, and the k_{min} are similar to those reported for homopolymers. The second point is the most important, as it modifies interpretations of protein hydrogen exchange studies[1] and of the random coil behavior of oxidized RNase.[31]

On the basis of the present work it clearly cannot be assumed that sites which share common structural properties, e.g., involvement in hydrogen bonding or three-dimensional positioning in an apolar environment, also share a common first-order exchange rate constant. Therefore, rate groups obtained by fitting the observed exchange curve with the smallest number of linear terms do not necessarily represent groups of sites with common structural properties. Also, it cannot be assumed[32] that a model which predicts a single rate of protein unfolding necessarily predicts a single rate of exchange. However, it is still possible to derive considerable information about the dynamics of protein conformation by combining exchange rate data from a native protein over a wide range of temperatures and pH's with data from perturbants which have a differential effect on the various regions of the molecule.[12]

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[1] Englander, S. W., in *Poly-α-amino Acids*, ed. G. Fasman (New York: Marcel Dekker, Inc., 1967), p. 341.
[2] Ikegami, A., and N. Kono, *J. Mol. Biol.*, **29**, 257 (1967).
[3] Englander, S. W., and A. Poulsen, *Biopolymers*, **7**, 579 (1969).
[4] Leichtling, B. H., and I. M. Klotz, *Biochemistry*, **5**, 4026 (1966).
[5] Neilsen, S. O., W. P. Bryan, and K. Millelsen, *Biochim. Biophys. Acta*, **42**, 550 (1960).
[6] Hirs, C. H. W., *J. Biol. Chem.*, **219**, 611 (1956).
[7] Jirgensons, B., *Biochim. Biophys. Acta*, **200**, 9 (1970).
[8] Ahmed, A. K., Ph.D. thesis, University of Minnesota (1969).
[9] Klee, W. A., and F. M. Richards, *J. Biol. Chem.*, **229**, 489 (1957).
[10] Rosenberg, A., and K. Chakravarti, *J. Biol. Chem.*, **243**, 5193 (1968).
[11] Englander, S. W., *Biochemistry*, **2**, 798 (1963).
[12] Woodward, C. K., and A. Rosenberg, submitted for publication.
[13] Harrington, W. F., and J. A. Shellman, *C. R. Trav. Lab. Carlsberg*, **30**, 21 (1956).
[14] Robinson, R. A., and R. H. Stokes, *Electrolyte Solutions* (London: Butterworths Publications, 1955).
[15] Hade, E. P., and C. Tanford, *J. Amer. Chem. Soc.*, **89**, 5034 (1967).
[16] Berger, A., A. Lowenstein, and S. Meiboom, *J. Amer. Chem. Soc.*, **81**, 62 (1959).

[17] Miller, W. G., and C. V. Goebel, *Biochemistry*, **7**, 3925 (1968).
[18] Hvidt, A., and S. O. Nielsen, *Advan. Protein Chem.*, **21**, 287 (1966).
[19] Bigelow, C. C., and I. I. Geschwind, *C. R. Trav. Lab., Carlsberg*, **31**, 283 (1960).
[20] Bigelow, C. C., and I. I. Geschwind, *C. R. Trav. Lab., Carlsberg*, **32**, 89 (1961).
[21] Bigelow, C. C., *C. R. Trav. Lab. Carlsberg*, **31**, 305 (1960).
[22] Harrington, W. F., and M. Sela, *Biochim. Biophys. Acta*, **31**, 427 (1959).
[23] White, F. H., Jr., *J. Biol. Chem.*, **236**, 1353 (1961).
[24] Young, D. M., and J. T. Potts, Jr., *J. Biol. Chem.*, **238**, 1995 (1963).
[25] Segal, D. M., and W. F. Harrington, *Biochemistry*, **6**, 768 (1967).
[26] Brants, J. F., and L. Hunt, *J. Amer. Chem. Soc.*, **89**, 4826 (1967).
[27] It should be pointed out that since the first measurement is at 3 ± 1 min, the zero point is a theoretical minimum and is assumed to equal at least the number of peptide nitrogens. That this is justified is shown in Fig. 1 by the curves at lower pH and the same temperature; at the pH_{min} the 2-min measurement gives 118–124H_{rem}. Any underestimate of zero time H's would tend to increase the curvature.
[28] Klotz, I. M., and D. D. Miller, *Biochemistry*, **8**, 12 (1969).
[29] Wishnia, A., and M. Saunders, *J. Amer. Chem. Soc.*, **84**, 4235 (1962).
[30] Rosenberg, A., and J. Enberg, *J. Biol. Chem.*, **244**, 6153 (1969).
[31] Tanford, C., *Advan. Protein Chem.*, **23**, 121 (1968).
[32] Hermans, J., L. Dennis, and D. Ferro, *Nature*, **224**, 175 (1969).