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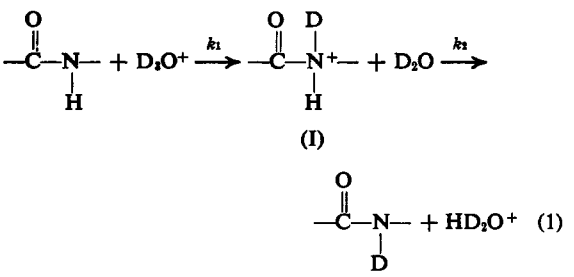
Local Environment Effects on Hydrogen–Deuterium Exchange*

Irving M. Klotz and Delbert D. Mueller†

ABSTRACT: The rate–pH profile of hydrogen–deuterium exchange of an amide NH in a polymer is shifted toward higher pH in the presence of an anionic detergent, sodium lauryl sulfate, which is bound by the polymer. This shift can be accounted for in terms of a change in local environment of the amide group which influences the acid–base character of the CONH moiety. This extrinsic effect is analogous to that produced intrinsically by electron-withdrawing substituents attached to an amide group. H–D exchange rates may be modified by perturbations of the activated state, as well as of the ground state, of the kinetic transition.

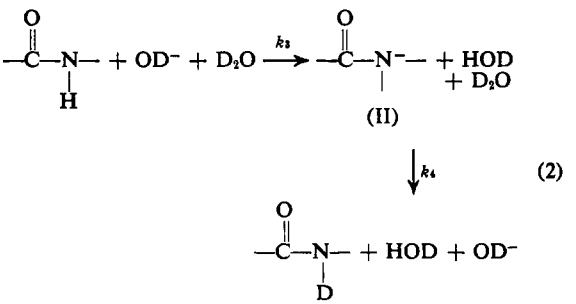
Hydrogen–deuterium exchange is a kinetic process. Thus, as in all rate processes, H–D interchange depends upon a transition from a ground state to an activated state (Figure 1). In studies with polypeptides and proteins it has generally been assumed that any attenuation in exchange rate is to be ascribed to a conformation of the macromolecule which lowers the energy of the ground state (Hvidt and Nielsen, 1966). Originally this state of lowered energy was assumed to be a helical structure. More recently masked conformations have been presumed to be inaccessible for less-defined steric reasons (Hvidt and Nielsen, 1966).

It is also obvious (Figure 1) that a change in the relative energy of the activated state, due to modifications in the environment in which an amide group may be situated, could likewise affect the kinetics of the isotopic exchange (Klotz, 1968).



(1)

Structural and environmental features that might affect the energy of the activated state reveal themselves when we examine the mechanism of the exchange reaction (Berger *et al.*, 1959; Klotz, 1968). In acid solution the steps are given by eq 1. In view of the weak basicity of the amide NH group one expects k_1 to be rate controlling in this acid-catalyzed reaction. In basic solution we may write



(2)

In view of the weak acidity of the amide NH group, k_3 should be rate limiting in the base-catalyzed reaction.

In either pathway a charged intermediate is formed, a cation (I) in the former, and an anion (II) in the latter. It seems very likely, therefore, that the activated state would also be a charged species.

Recent experiments with a synthetic polymer (Scarpa *et al.*, 1967), polyisopropylacrylamide, have shown that slow hydrogen–deuterium exchange may be observed for amide NH groups attached to a flexible, swollen polymer that is not in a compact conformation. These

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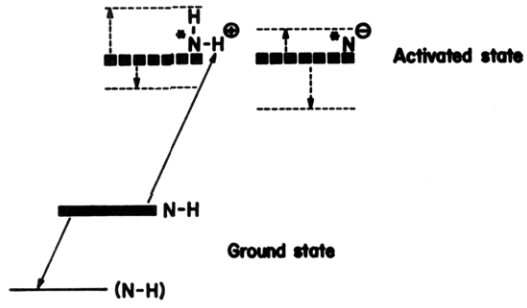

FIGURE 1: Schematic diagram of possible variations in energy of ground and activated states in hydrogen–deuterium-exchange reactions.

observations are readily interpreted in terms of the mechanism illustrated in eq 1 and 2. In the polymeric matrix, charge species should be relatively unfavored since the immediate surroundings are apolar in nature (Figure 2). Under these circumstances one would expect the rate constants, k_1 and k_3, to be diminished in magnitude, for the relative energy (Figure 1) of the activated species would be higher in the polymeric environment.

In essence the behavior of the polymer indicates that the local environment of a CONH group is of crucial importance in controlling the rate of exchange. It follows, therefore, that if we could change the local environment in some clearly defined way, we should also change the kinetics of the hydrogen-deuterium interchange in a controlled fashion.

It is well known that detergent ions are bound to polymers as well as to proteins (Karush and Sonenberg, 1949; Saito, 1958; Aoki and Foster, 1958). It seemed of interest, therefore, to examine the effect of an anionic detergent, sodium dodecyl sulfate, on the exchange characteristics of amide groups in polyisopropylacrylamide. This anionic detergent would, when bound to the polymer, place a negative charge in the neighborhood of the amide group (Figure 2). Such a charge should stabilize the cationic species (I) which is the intermediate in the acid-catalyzed reaction and should destabilize the anionic species (II) which is the intermediate in the base-catalyzed reaction. Under these circumstances, one would expect the entire parabolic rate–pH profile (Klotz, 1968) to be shifted sidewise toward higher pH values. This is a clearly defined prediction which can readily be examined experimentally.

Experimental Methods

Materials. *N*-Isopropylacrylamide was obtained from the American Cyanamid Co. and was recrystallized from a toluene–hexane mixed solvent. Sodium dodecyl sulfate was purchased from Mann Laboratories and was used without further purification. Heavy water was supplied by Bio-Rad Laboratories and was warranted to be 99.84% D_2O.

Poly-*N*-isopropylacrylamide was prepared from the monomer by a free-radical polymerization route in aqueous solution as described previously (Scarpa *et al.*, 1967). Its intrinsic viscosity, in aqueous solution

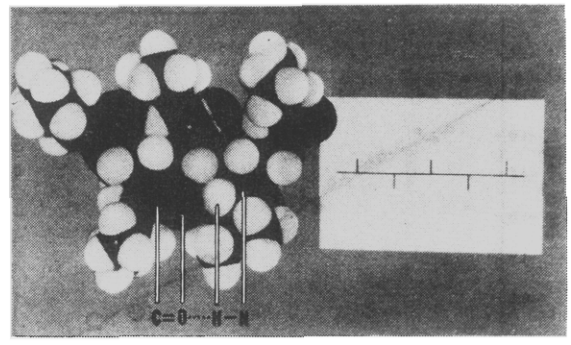

FIGURE 2: Atomic model of segment of polyisopropylacrylamide containing five residues. Black = carbon; dark gray = oxygen; white = hydrogen; light gray = nitrogen. The card at the right shows the spatial disposition of the side chains of the five residues.

at 15°, was 3.5 dl/g, as compared to 3.0 for the product of Scarpa *et al.* (1967). The degree of polymerization of the two preparations thus must be similar. Hydrogen–deuterium exchange rates of the two materials at pD 4.68 and 5.80 agreed within 20%.

Rate Measurements. A volume of D_2O containing 0.020 M buffer and the correct amount of sodium dodecyl sulfate was adjusted to the desired pH with either DCl or NaOD solutions. Acetate was used for pH 5.0–5.7, cacodylate for 5.7–7.0, and phosphate for 7.0–8.0. pH measurements were made with a Radiometer Model 4 pH meter equipped with a Corning semimicrocombination electrode. Spectrophotometer cells (5 cm) with D_2O solution were placed in the cell compartment and cooled to 15°. Once at the proper temperature the D_2O solution was transferred to a small container into which was added an exact amount of polymer. Dissolution was carried out in a cold room at 5°, with the aid of a modified Cole-Parmer Supermixer. The solution was subsequently centrifuged to remove foam and returned to the sample cell through a glass wool plug.

For the determination of the effect of sodium dodecyl sulfate the ratio of polymer to detergent was held constant and the pH was varied. All kinetic runs were made with 2% polymer concentration. Exchange rates were obtained by following the intensity of the overtone infrared absorption band of HOD at 1.41 μ using a Cary 14 spectrophotometer equipped with a 0–0.1 absorbance slide wire. The HOD absorbance was measured relative to that at 1.24 μ, an invariant region of the near-infrared spectrum, to correct for any variations from sample to sample due to differences in light scattering.[1]

Binding Measurements. A few equilibrium dialysis experiments (Klotz *et al.*, 1946) were carried out with poly-

[1] As in the previous sample of polyisopropylacrylamide (Scarpa *et al.*, 1967) some turbidity was observed with 2% solutions of polymer. This turbidity is not removed by centrifugation. Equilibrium ultracentrifugation gave a molecular weight somewhat over 200,000. In 2% polymer solutions containing only 0.1% dodecyl sulfate, light scattering was markedly reduced (probably because of electrostatic effects of the bound anions) but the rate constants for hydrogen–deuterium exchange were affected only slightly.

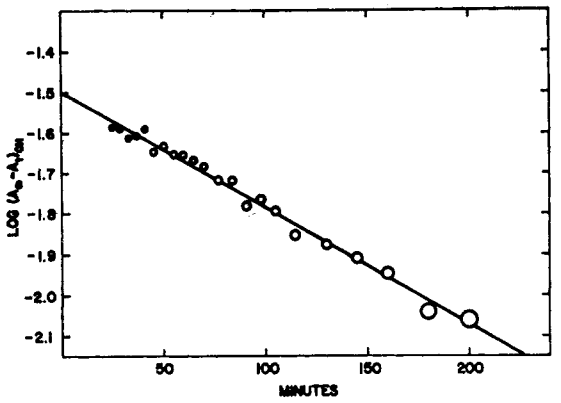

FIGURE 3: Typical first-order rate plot for NH → ND exchange at pD 5.72 for polyisopropylacrylamide in D_2O in the presence of 0.0346 M sodium dodecyl sulfate: $k = 6.59 \times 10^{-3}$ min^{-1}, $t_{1/2} = 105$ min.

mer and methyl orange to assure ourselves that polyisopropylacrylamide does bind large organic anions to some extent. Phosphate buffer (pH 6.9) was used in the aqueous solvent. Polymer concentration was varied from 1 to 2%, and methyl orange from about 10^{-5} to 10^{-4} M. We are indebted to Miss Virginia Stryker for her assistance in carrying out these experiments.

Results

As described previously (Scarpa *et al.*, 1967), pseudo-first-order rate constants for the reaction

$$>NH + D_2O \xrightarrow{k} >ND + HOD \qquad (3)$$

may be calculated from the equation

$$-\frac{d(NH)}{dt} = \frac{d(OH)}{dt} = k(NH) \qquad (4)$$

since D_2O is present in overwhelming excess. It has been shown previously (Scarpa *et al.*, 1967) that exchange in polyisopropylacrylamide alone in aqueous solution does follow first-order kinetics. Figure 3 illustrates that first-order kinetics is also obtained for the polymer (at 2% concentration) in the presence of 1% sodium dodecyl sulfate.[1]

The dependence of the first-order rate constant upon pD is shown in Figure 4, for the polymer itself and for polymer in the presence of sodium dodecyl sulfate. It is apparent that pD_{min}, the pD of lowest exchange rate, is shifted substantially to higher pH but that k_{min}, the rate constant at pD_{min}, is changed to a much smaller extent.

The binding measurements indicate that approximately 0.1 mole of methyl orange is bound per 10^5 g of polymer at a free dye concentration of 10^{-4} M. Since sodium dodecyl sulfate is bound much more strongly than methyl orange anions (Klotz *et al.*, 1946; Karush and Sonenberg, 1949), it is apparent that the polymer can bind substantial quantities of the detergent.

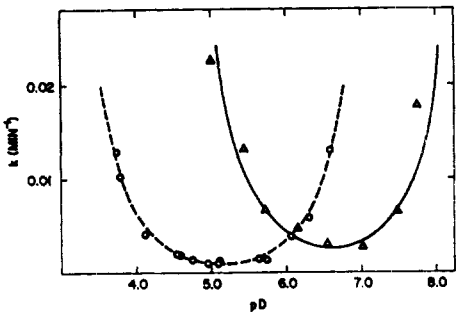

FIGURE 4: Rate–pH profile for polyisopropylacrylamide in D_2O at 15° in the absence (○) and in the presence (△) of 0.035 M sodium dodecyl sulfate. Polymer concentration is 0.18 residue molar.

Discussion

First-Order Kinetics. An any given moment, some amide groups have sodium dodecyl sulfate in the vicinity and others do not. Nevertheless, there is no kinetic evidence of two classes of exchanging amide NH's; the rates of exchange follow simple first-order kinetics (Figure 3). We may conclude, therefore, that the binding of detergent is fast compared to the rate of H–D exchange and that the time-average environment of every CONH group is the same.

pH Dependence. Despite the similarity in the order of the process, exchange shows a markedly shifted pH profile in the presence of sodium dodecyl sulfate (Figure 4). pD_{min} is shifted by 1.5 units, *i.e.*, (D^+_{min}) is changed by a factor of 30. On the other hand, k_{min} is changed by only a factor of 3.

This marked difference in sensitivity of (D^+_{min}) and k_{min} to perturbations in the environment of the CONH group can be readily understood on the basis of the mechanism of the H–D-exchange reaction if Brønsted-type concepts are adapted to this situation.

As has been shown previously (Leichtling and Klotz, 1966) since the exchange reaction is catalyzed by both H^+ (or D^+) and OH^- (or OD^-), one may write the following equation for the rate constant, k

$$k = k_O + k_D(D^+) + k_{OD}(OD^-) \qquad (5)$$

where k_D and k_{OD} are the catalytic constants for the respective species and k_O represents the rate constant for the "spontaneous" reaction due to solvent itself. A few simple algebraic steps (Leightling and Klotz, 1966) lead to an expression for (D^+_{min}), the deuterium ion concentration at which k reaches a minimum

$$(D^+_{min}) = \left(\frac{k_{OD}}{k_D}K_W\right)^{1/2} \qquad (6)$$

where K_W is the self-ionization constant of water.

We can see from eq 6 that to change (D^+_{min}) we must change either or both catalytic constants, k_D and k_{OD}. (If the character of the solvent water changes, then K_W may also change, but for the present we shall assume that this is not being perturbed.) Looking at the mech-

anism of the acid-catalyzed exchange, eq 1, we see that any factor which weakens the basicity of the >NH would decrease the rate of protonation. Using a Brønsted type of approach we may state a quantitative formulation as

$$k_D = bK^{-\beta} \qquad 0 < \beta < 1 \tag{7}$$

where b and β are constants for the amide group in which the acidity constant K for the >NH is being varied by changes in the environment. In a corresponding fashion, looking at the mechanism of the base-catalyzed reaction, eq 2, we conclude that any factor that weakens the basicity of the >NH will increase the tendency of this group to give up its proton and hence increase k_{OD}. Thus we may write

$$k_{OD} = aK^{\alpha} \qquad 0 < \alpha < 1 \tag{8}$$

where a and α are constants. If we insert eq 7 and 8 into eq 6 we find that

$$(D^+_{min}) = \left(\frac{a}{b}K_W\right)^{1/2} K^{(\alpha+\beta)/2} \tag{9}$$

or

$$(pD_{min}) = \text{constant} + \frac{\alpha + \beta}{2}pK \tag{10}$$

Turning to k_{min}, we may start with the equation (Leichtling and Klotz, 1966)

$$k_{min} = k_O + 2(k_D k_{OD} K_W)^{1/2} \tag{11}$$

Again inserting eq 7 and 8 we obtain

$$k_{min} = k_O + 2(abK_W)^{1/2} K^{(\alpha-\beta)/2} \tag{12}$$

Comparing eq 9 and 12 we see that (D^+_{min}) should respond strongly to changes in K of the NH moiety since it depends exponentially upon the *sum* $(\alpha + \beta)$ but that there should be only a mild dependence of k_{min} on K since it depends exponentially upon the *difference* $(\alpha - \beta)$. The experimental observations of the kinetics in the presence of sodium lauryl sulfate definitely fit this prediction. First, the shift in pD_{min} is in the direction to be expected from the effect of a neighboring negative charge on the pK of the >NH group. In addition the change in (D^+_{min}) in the presence of anionic detergent is large (about 30-fold) as one expects from the $(\alpha + \beta)/2$ exponent in eq 9, whereas the change in k_{min} (about threefold) is much smaller, in line with the appearance of $(\alpha - \beta)/2$ as the exponent in eq 12.

Thus Brønsted-type concepts account qualitatively for the effect of anionic detergent on the exchange characteristics of the polymer. This approach focuses on the charged intermediates (I and II of eq 1 and 2) of the activated state (Figure 1). As has been pointed out previously (Klotz, 1968), we must also keep in mind that these cationic and anionic intermediates, $CONHD^+$ and CON^-, are obtained by the transfer of a proton from or to a water molecule in the vicinity of the amide group. In the apolar environment of the polymer surface (see Figure 2) the character of the water would be different than in bulk water. One might expect water at the surface of this macromolecule to behave as in a mixed organic–aqueous solvent (*e.g.*, dioxane–H_2O) or perhaps to form ice-like clathrates around apolar groups (Klotz, 1960). In either event, K_W, the self-dissociation constant of H_2O, should be lowered, if we may judge from the known behavior of K_W in dioxane–water mixtures (Harned and Owen, 1958) or in normal ice (Eigen and De Maeyer, 1958). If K_W is lowered, in effect the energy of the charged activated states is raised. This seems to be a major contribution to the alteration of exchange behavior of the polymer itself. However, it seems unlikely that this effect is greatly accentuated by addition of detergent, for if it were one would expect to find a further decrease in k_{min} (see eq 12) whereas experimentally k_{min} increases threefold. Thus the Brønsted type of interaction must be primarily responsible for the observed perturbations by the detergent.

Conclusion

It is thus apparent that the local environment of the CONH group determines its hydrogen–deuterium-exchange kinetics. Modifications of the local environment may affect the rate constant k by perturbing either the ground state or the activated state (Figure 1) of the CONH moiety. A typical example of an effect on the ground state is the formation of an N—H···O=C bond. A corresponding illustration of an effect on the activated state is provided by the results with sodium dodecyl sulfate.

It is also illuminating to categorize the many factors that have now been shown to affect hydrogen–deuterium exchange rates as either *intrinsic* or *extrinsic* factors (or autoplastic and alloplastic effects; Klotz, 1966). Intrinsic factors, in addition to hydrogen bonds, are illustrated by inductive effects of groups adjacent to the CONH. As has been shown (Leichtling and Klotz, 1966) electron-withdrawing substituents on either side of the CONH affect k_{min} and pD_{min}, in a predictable direction. Extrinsic factors, in turn, are illustrated in the catalytic effects of H^+, OH^-, and other general acids and bases, as well as by the effect of additives such as sodium dodecyl sulfate, or of nonpolar materials such as dioxane.

Thus it is now possible to change the rates of hydrogen–deuterium exchange in an amide group, in a reasonably predictable manner, upward, downward, or sideways.

Acknowledgment

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Peptide-Bond Hydrolysis Equilibria in Native Proteins. Conversion of Virgin into Modified Soybean Trypsin Inhibitor*

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ABSTRACT: Virgin (Arg(64)–Ile(65) bond intact) and modified (Arg(64)–Ile(65) bond cleaved) soybean trypsin inhibitor were separated by preparative disc gel electrophoresis. Incubation of *either* the virgin or the modified inhibitor with catalytic quantities of trypsin at pH 4.00 and 20° yields the *same* equilibrium mixture containing 86 ± 2% of modified and 14 ± 2% of virgin inhibitor as judged by analytical disc gel electrophoresis. Thus, the existence of an equilibrium between these two forms is conclusively demonstrated. Preliminary values of the equilibrium constant as a function of pH were obtained. As expected, there is a broad minimum ($K_{hydrolysis} \sim 2$) in the pH 5–8 range and sharp increases at both high and low pH values.

Incubation of virgin soybean trypsin inhibitor with catalytic quantities of trypsin converts it into modified inhibitor by hydrolysis of the Arg(64)–Ile(65) peptide bond (Figure 1) (Finkenstadt and Laskowski, 1965; Ozawa and Laskowski, 1966). In previous papers we have implied that this reaction does not lead to the complete conversion of virgin to modified inhibitor but rather that an appreciable amount of virgin inhibitor remains after the system has reached equilibrium. We have felt that this point was of sufficient importance to deserve proof and that only a demonstration that both pure virgin inhibitor and pure modified inhibitor are converted by catalytic amounts of trypsin to the same equilibrium mixture would serve as such a proof. This was achieved by employing disc gel electrophoresis (Ornstein, 1964; Davis, 1964) both as a preparative method for obtaining pure modified soybean trypsin inhibitor and as an analytical technique for monitoring the composition of the reaction mixtures.

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† National Institutes of Health Predoctoral Fellow.

Experimental Procedure

Materials and Methods. Virgin soybean trypsin inhibitor (special grade, lot B 7303) (selected after extensive purity testing of commercially available lots) was obtained from Gallard-Schlesinger Chemical Corp. Some of this material was converted to an equilibrium mixture of virgin and modified inhibitors according to the method of Ozawa and Laskowski (1966). Bovine trypsin (EC 3.4.4.4) (lots TRL71C and TRL7FA) was obtained from Worthington Biochemical Corp. Glycine, acrylamide, *N,N'*-methylenebisacrylamide, *N,N,N'-N'*-tetramethylethylenediamine, and Napthol Blue Black were purchased from Eastman Organic Chemicals. Sephadex G-200 was obtained from Pharmacia Fine Chemicals. Tris (primary standard) was purchased from Fisher Scientific Co. *p*-Nitrophenyl *p*-guanidinobenzoate hydrochloride (lot K-5965) was purchased from Cyclo Chemical Corp. All other chemicals were reagent grade.

All pH measurements were made using a Radiometer pH meter (Model TTT1A). All protein concentrations were determined with a Cary Model 14 spectrophotometer. Optical factors (at 280 mμ) used were 0.651 mg ml^{-1} (OD unit)$^{-1}$ for trypsin (Worthington, 1967) and 1.1 mg