Tritium–Hydrogen Exchange Studies of Protein Models. I. Gramicidin S-A*

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ABSTRACT: Tritium–hydrogen exchange experiments, using a new rapid dialysis technique, have been performed on the cyclic decapeptide gramicidin S-A in aqueous solution. The technique, countercurrent dialysis, was found to give highly accurate results and should have general applicability to exchange measurements. The molecule has eight exchangeable peptide hydrogens, all of which may be followed in the pH_{min} region. Slowly exchanging peptide hydrogens are clearly present. In the base-catalyzed region, there are four rapidly exchanging hydrogens and two groups of two slow hydrogens each, the slowest of which exchange more than an order of magnitude more slowly than those of poly-DL-alanine. In the acid-catalyzed region, there are again four slow hydrogens, but they are found in one kinetic class. The results for the pH dependence of the exchange rate are consistent with an EX_2 mechanism of exchange. This fact constitutes important evidence for the validity of this mechanism since gramicidin S-A, with respect to exchange, is a much simpler system than a protein. The free energies of the opening reactions are calculated to be about 0.483 and 1.73 kcal per mole for the two slow classes in the base-catalyzed region. Since the energy of the molecule has been estimated to be of the order of −100 kcal/mole, it is clear that the conformational changes needed to allow the slow hydrogens to exchange are not major ones and perhaps involve only small rotations about bonds. Our data for exchange in aqueous solution are found to agree quite well with nuclear magnetic resonance data obtained in methanol and dimethyl sulfoxide, thus implying that the primary conformational determinants of this molecule are steric effects and backbone energetics. Finally, the results are shown to be consistent with one of the possible models of gramicidin S-A proposed earlier by Hodgkin and Oughton (1957) (Hodgkin, D. C., and Oughton, B. M. (1957), *Biochem. J. 65*, 752) or by Schwyzer and Sieber (1957) (Schwyzer, R., and Sieber, P. (1957), *Helv. Chem. Acta 15*, 624) and the refinement of this model by Stern *et al.* (1968) (Stern, A., Gibbons, W., and Craig, L. C. (1968), *Proc. Natl. Acad. Sci. 61*, 734.

The hydrogen exchange technique has considerable potential for increasing our knowledge of protein structure and interactions. Recent use of radioisotope methodology (Englander, 1963; Segals and Harrington, 1967) has enabled reasonably accurate data to be obtained for proteins. Furthermore a considerable amount of information on model compounds such as simple amides (Berger *et al.*, 1959; Neilsen, 1960; Klotz and Frank, 1962, 1964), oligopeptides (Neilsen *et al.*, 1960), and polypeptides (Bryan and Neilsen, 1960; Englander, 1967; Leichtling and Klotz, 1966) is available. However, as with other physicochemical techniques, the central problem is to extrapolate the results from model compounds to the complex three-dimensional structure of a protein in solution. To aid in bridging this gap, we have undertaken a study of several small, naturally occurring peptides. Some of these compounds may be considered protein models in that they possess both a discrete tertiary structure and a biological function. Gramicidin S-A is of particular interest not only from the standpoint of hydrogen exchange, but also as the object of much current speculation regarding its most probable conformation. With regard to the former, we shall deal with the validity of the EX_2 mechanism of exchange (Hvidt and Neilsen, 1966) and the possible nature of the "opening" mechanisms. Since much of the recent exchange literature assumes this mechanism to be valid, an experimental confirmation is essential. The use of proteins for this purpose may be hazardous since they contain hundreds of exchangeable hydrogens, some of which may belong to buried side chains or side-chain hydrogen bonds as well as an "infinitely" slow class. Such difficulties are not present in the case of gramicidin S-A, which has only eight hydrogens exchanging at observable rates, all of which may be shown to belong to the peptide groups involved. The primary structure of gramicidin S-A is known to be

┌LVal-LOrn-LLeu-DPhe-LPro-LVal-LOrn-LLeu-DPhe-LPro┐
└──┘

Over the pH range covered by these experiments, the ornithine side chains are virtually all in the charged form. In regard to the conformation of gramicidin S-A, our experiments provide informative physicochemical data for the molecule in aqueous solution which allows a choice from the possible models of the conformation.

Materials

Gramicidin S-A was isolated from a crude preparation by countercurrent distribution and lyophilized from glacial acetic acid (Craig *et al.*, 1950). Tritiated water was obtained from the New England Nuclear Corp. All materials used in the buffers were reagent grade.

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Methods

Tritium–hydrogen exchange measurements of exchange-out are generally accomplished in three steps: (1) tritiation of a solution of the macromolecule of interest; (2) separation of the bulk tritiated solvent from the macromolecule to give a "pool" of tritiated material; and (3) withdrawal of aliquots from the pool at various times. "Exchanged-out" tritium is then removed, leaving only the tritiated molecule, whose concentration and degree of labeling is then determined.

In the experiments reported here, 2–4 mg/ml of gramicidin S-A was labeled to the extent of about 5 mCi/ml with tritiated water. The solution was set aside for several days, allowing attainment of isotopic equilibrium and completion of step one. Steps two and three were accomplished by a method formally analogous to the two-column Sephadex technique (Englander, 1963). However, the peptide is too small for the usual gels and neither Sephadex G-10 nor Bio-Gel P-2 was suitable due to adsorption of the peptide. A dialysis method was sought. Previously published methods (Englander and Crowe, 1965) required such a long contact time between membrane and solution that the small peptide would dialyze through the membrane by the time tritium clearance was achieved. The countercurrent dialysis method (Craig and Stewart, 1965) in its improved form (Craig *et al.*, 1968), however, was found to be efficient enough to remove the tritiated water and yet allow retention of easily detectable quantities of gramicidin S-A. A "long" countercurrent dialysis column, 90 cm in length, was used to separate bulk solvent from the tritiated peptide. A similar "short" dialysis column, 45 cm in length, was used for separation of the exchanged-out tritium from the pooled material. Concentrations of the final samples were determined in a Zeiss PMQ II spectrophotometer at 225 mμ. The extinction at this wavelength followed Beer's law over the concentration range of interest. Sample (500 μl) was then quickly pipetted into a glass scintillation vial containing 10 ml of Bray's solution and counting was done in a Packard Tri-Carb liquid scintillation counter.

Optimization of Clearance and Retention. It was desired to dialyze away a maximum amount of tritiated water while losing a minimum of the peptide. Since the extent of dialysis in the apparatus is directly dependent upon the diffusate outflow rate and the membrane porosity, and inversely dependent upon the retentate inflow rate, these parameters required an optimal adjustment. Retentate inflow for each column was controlled by a Holter bilateral roller pump, Model RDO44 (Holter Co., Bridgeport, Pa.). Diffusate outflow was controlled with Manostat Teflon valves and driven by gravity. Membrane porosity was adjusted *via* the technique of acetylation (Craig and Konigsberg, 1961). The long column membrane, *after* installation on the column, was acetylated with 25% acetic anhydride in pyridine at room temperature for about 5 hr, while that of the small column was treated similarly for 4 hr. With this porosity, the required flow rates for the long column were 0.2 and 10 ml per min, for retentate and diffusate, respectively. The analogous flow rates for the short column were 0.3 and 6 ml per min.

When arranged as described, the long column was able to dialyze away in excess of six orders of magnitude of tritiated water while retaining 50–60% of the gramicidin S-A applied. The short column could clear in excess of three orders of magnitude of tritiated water while retaining 70% of the peptide. With the initial degree of labeling employed, only several hundred counts per minute per milliliter of contaminating tritiated water emerged from the long column, compared with over 10,000 cpm/ml in the peptide. Consequently, the very early time points (<25 min), which were obtained directly from the long column, contained an error of the order of several per cent, while the experimental points coming from the two-column technique had an insignificant error all attributable to contamination.

Experimental Procedure. All experiments were performed in a specially constructed refrigerated box maintained at 4°. Columns could be maintained at 0.0 ± 0.5° by the use of cylindrical glass jackets running the length of the column and connected to an ethylene glycol–water constant-temperature bath cooled by a separate refrigeration unit.

In a typical experiment, 1 ml of tritiated gramicidin S-A solution was passed through the long column at the flow rate specified above. The diffusate was collected in a tightly sealed glass bottle and discarded. Emerging fractions of retentate were collected in glass tubes immersed in ice. The position of the peak was ascertained by sampling the optical density of the fractions at 225 mμ. Peak tubes were pooled in a screw-cap glass tube and kept on ice, frequently replaced, for the remainder of the experiment. At intervals, 0.2-ml samples were removed from the pool tube, passed through the short column, and assayed. By judicious overlapping and repetition of experiments, a satisfactory number of points could be obtained. Since the elapsed time was carefully monitored, it was possible to determine the number of hydrogens remaining on the molecule as a function of time. However, to correct for time at the start and end of the experiment when there is no effective exchange (time in tubing, capillary, plus one-sixth of column transit time), a zero-time correction is necessary. It was assumed that exchange would become unidirectional outward when 90%, *i.e.*, one order of magnitude, of the bulk tritiated water was removed. Since clearance is roughly proportional to the distance traveled up the column (Craig and Stewart, 1965), zero time was taken as the amount of time for the solute to travel one-sixth of the way up the long column. This time interval was easily determined by following the progress of a pulse of concentrated Blue Dextran solution through the apparatus. The zero-time correction was about 9 min. Since many of the experiments were over 1000-min duration, this correction was not critical for the extrapolations.

Results

The results of exchange experiments conducted at eight different pH values are shown in Figure 1. Hydrogens remaining, per molecule, are plotted against time on a semilogarithmic scale. Theoretical curves, obtained

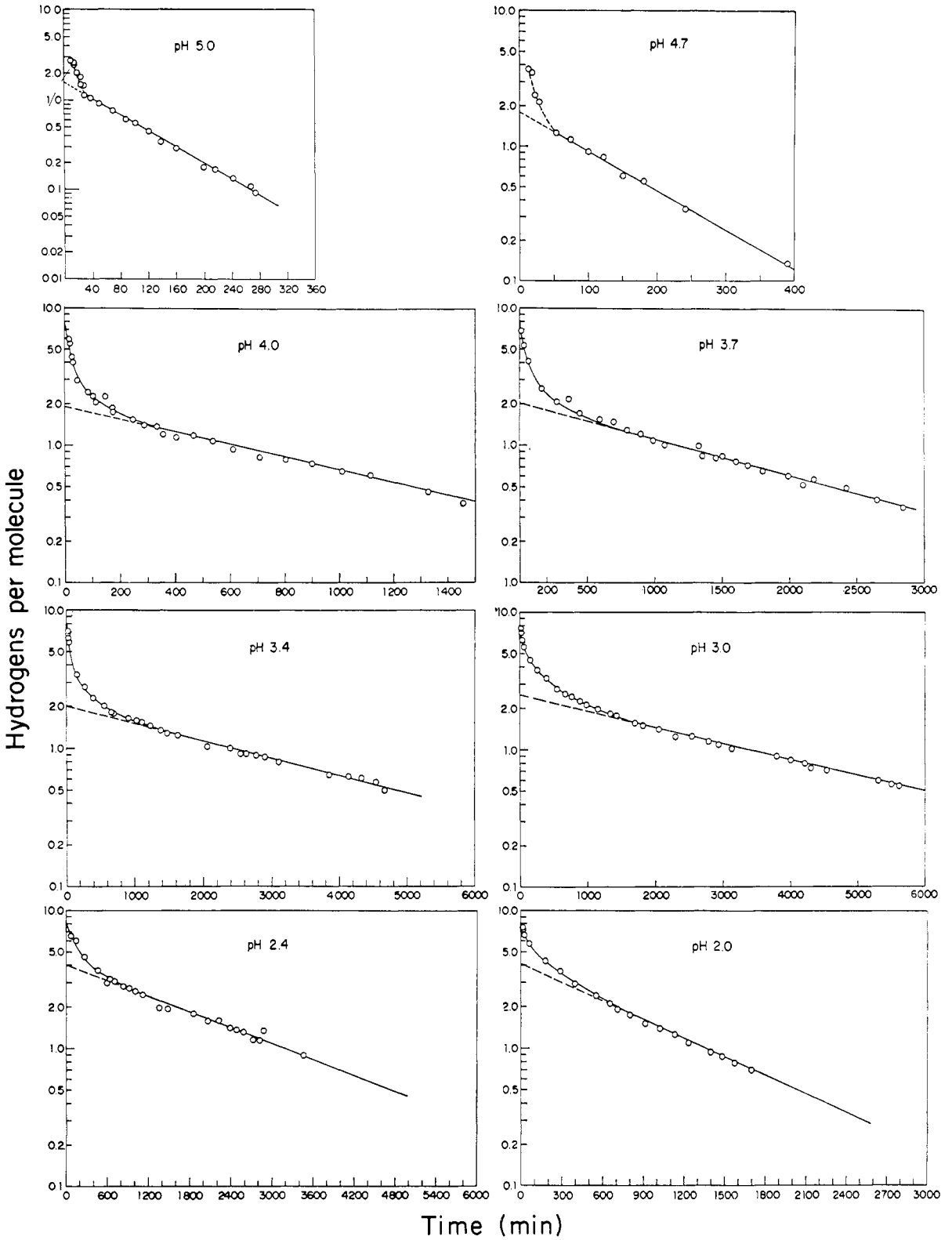

FIGURE 1: Tritium–hydrogen exchange-out data at 0° and various pH values: pH 5.0, 0.01 M acetate; pH 4.7, 0.01 M acetate; pH 4.0, 0.01 M formate; pH 3.7, 0.01 M formate; pH 3.4, 0.01 M formate; pH 3.0, 0.001 M formate; pH 2.4, 0.01 M phosphate; and pH 2.0, 0.01 M phosphate. With the exception of pH 3.0 (see text), the solid curves through the experimental points are computer drawn and based on the rate constants and class sizes depicted in Table I. The dashed lines are extrapolations from the linear portions of the curves to zero time.

as described below, are drawn through the points and zero-time extrapolations made. It can be seen that in the base-catalyzed region, *i.e.*, above pH 3.0, the extrapolations cluster about two very slowly exchanging hydrogens per molecule, while in the acid-catalyzed region, below pH 3.0, four are found. It should be noted that below pH 4.0, where the exchange has slowed sufficiently, the earliest points are very close to the theoretical value of eight hydrogens per molecule. This is clearly seen in the minimum region, around pH 3.0. Thus, it would appear unlikely that "bound water" is involved in the exchange or that an equilibrium isotope effect is operative to a significant extent.

Analysis of Curves. All hydrogen exchange curves may be formulated from the relation (Hvidt and Neilsen, 1966)

$$H(t) = \sum_i c_i e^{-k_i t}$$

where $H(t)$ is the number of hydrogens remaining at time t, per molecule; c_i is the number of hydrogens in the ith kinetic class; k_i is the rate constant of exchange for the ith kinetic class; and t is time.

It is well known that such exchange curves may be difficult to analyze (Linderstrøm-Lang, 1955). Most difficulties stem from two sources: the length of the experiment may be insufficient to allow accurate determination of the slope of the terminal region, or the rate constants may be of similar magnitude and therefore not resolvable. In the case of the present experiments, the former difficulty was largely avoided by following the apparent slow class of hydrogens over a period of several half-lives. The latter problem was not serious for the base-catalyzed region, since the rate constants for the three kinetic classes differed by about an order of magnitude. For the acid-catalyzed region, the four rapidly exchanging hydrogens appeared to belong to two classes with rate constants of similar magnitude. Consequently, in one case (pH 2.4) the analysis was not unique for the early portion of the curve and classes I and II could be combined into a single class of rate constant 5.13×10^{-3} min^{-1} with only slightly poorer fit of the data. In the minimum region (pH 3.0), the nonintegral size of the very slow class also precluded a unique analysis. However, in all cases, the rate of exchange of the very slow hydrogens and the extrapolation to zero time was clear. This enabled us to analyze the curves by the method of successive subtractions. The results of the analysis are summarized in Table I. Theoretical exchange curves were then generated from the class sizes and rate constants with the aid of a Control Data 160G computer and plotted in Figure 1. In the base-catalyzed region, class I will be referred to as the rapid class and class II as the intermediate in exchange rate. In the acid-catalyzed region, class I and class II are both rapidly exchanging. Class III will always be referred to as the very slow class of hydrogens.

Discussion

The rate of exchange of a given peptide hydrogen is known to be determined by at least three factors (Hvidt and Neilsen, 1966; Englander, 1967): (1) the distance of the experimental pH from the characteristic pH minimum of exchange, (2) the experimental temperature, and (3) structural effects (hydrogen bonding, inaccessibility, or steric effects).

The object of most hydrogen exchange experiments is to determine the effect of factor 3. But since all of the above will simultaneously influence the course of an exchange reaction, factors 1 and 2 must be accounted for first. Fortunately, extensive studies of simple amides, oligopeptides, and structureless polypeptides, referred to previously, have clearly defined the role of pH and temperature in hydrogen exchange. For poly-DL-alanine, it has been shown that (Hvidt, 1964)

$$\left(\frac{\partial \log k_3}{\partial T}\right)_{pH} \sim 0.05 \qquad (1)$$

TABLE I: First-Order Rate Constants (k, min^{-1}) and Class Sizes (c, hydrogens per molecule) for the Exchange of Gramicidin S-A at 0° and Various pH Values.

	Class I		Class II		Class III	
pH	k	c	k	c	k	c
			Base-Catalyzed Region			
5.0					1.07×10^{-2}	1.6
4.7					6.07×10^{-3}	1.8
4.0	5.78×10^{-2}	4.3	1.22×10^{-2}	1.8	1.05×10^{-3}	1.9
3.7	2.89×10^{-2}	4.0	6.19×10^{-3}	2.0	5.92×10^{-4}	2.0
3.4	2.31×10^{-2}	4.0	3.47×10^{-3}	2.0	2.86×10^{-4}	2.0
			Minimum Region			
3.0					2.67×10^{-4}	2.5
			Acid-Catalyzed Region			
2.4	9.24×10^{-3}	2.0	3.77×10^{-3}	2.0	4.33×10^{-4}	4.0
2.0	3.15×10^{-2}	2.0	4.62×10^{-3}	2.0	1.02×10^{-3}	4.0

$$\left(\frac{\partial \log k_3}{\partial \mathrm{pH}}\right)_T \sim 1 \qquad (2)$$

where k_3 is the rate constant of exchange and T is the temperature in degrees centigrade. Equation 2 holds only when the experimental pH is about one unit removed from the pH_{min}, depending upon the sharpness of the minimum in the pH–rate curve. Failure to take the role of pH and temperature into consideration probably accounts for the failure of previous attempts to find slowly exchanging hydrogens in gramicidin S-A (Balasubramanian, 1967). For example, the infrared experiments of Balasubramanian were carried out in pure D_2O at room temperature, implying pD $\sim$ 7 and about 20°. Our data gives a half-life of 2600 min to the slowest hydrogens of gramicidin S-A at 0° and in the neighborhood of the pH_{min}. From eq 1 and 2 we see that the exchange should proceed about 100,000 times faster at neutral pH and 20°, making the "slow" hydrogens unobservable under these conditions.

Hydrogens exchange slowly only in the relative sense, *i.e.*, slower than those of the appropriate model compound. In view of the work on polypeptide hydrogen exchange (Leichtling and Klotz, 1966), it is clear that the proper model compound is a structureless one with the same pH_{min}. The exchange rate for such a compound at a given temperature and pH would correct for factors 1 and 2. If any hydrogens in the molecule under investigation exchange more slowly than this, factor 3 must be responsible.

Since gramicidin S-A appears, from Table I, to have a minimum rate of exchange around pH 3.0, poly-DL-alanine, whose pH_{min} is similarly located, is the model of choice (Bryan and Neilsen, 1960). We have extracted rate constants from the tritium–hydrogen exchange data of Englander (1967) for poly-DL-alanine at 4°, corrected them to 0°, and inserted the values obtained into Table II. Since eq 2 is valid at pH 4.0 and 5.0, rate constants for these pH values were calculated from Englander's pH 4.7 data and also entered in the table. For the acid-catalyzed region, no tritium–hydrogen exchange data were available. However, deuterium–hydrogen exchange data (Bryan and Neilson, 1960) corrected to 0° gives rate constants of 2.94×10^{-2} and 7.28×10^{-2} for pH 2.37 and 2.07, respectively. While these numbers are not strictly comparable with our tritium exchange data, they are of the order of magnitude expected for the intrinsic rate of exchange of the unhindered peptide hydrogen in the acid-catalyzed region.

Comparison of our data for gramicidin S-A, in the base-catalyzed region, with that shown in Table II for poly-DL-alanine, shows that gramicidin S-A does, indeed, have slowly exchanging hydrogens. There are clearly two very slow hydrogens (class III) which exchange more than an order of magnitude more slowly than those of poly-DL-alanine. Inspection of the exchange curves shows a rapidly falling initial region followed by a more gently sloping region. Curve analysis shows that these two regions represent four and two hydrogens, respectively. The faster hydrogens (class I) are somewhat faster than those of poly-DL-alanine while the slower ones (class II) are several times slower. There are, then, four hydrogens which exchange more slowly than those of poly-DL-alanine, *i.e.*, four "slow" hydrogens. In the acid-catalyzed region, there are again four slow hydrogens. However, they are now all found in the same kinetic class (class III). Furthermore, the four faster hydrogens have probably separated into two kinetic classes.

Table I also reveals that, in the base-catalyzed region, between pH 3.7 and 5.0, the rate of exchange is in general linearly dependent on the hydroxyl ion concentration for all classes. While the pH 4.7 data deviated somewhat, the general trend is clear. For example, the change in exchange rate of the very slow class between pH 4.0 and 5.0 is almost precisely the factor of 10 predicted by eq 2. As will be shown below, this allows determination of the mechanism of exchange in gramicidin S-A.

The current and generally employed model for slow hydrogen exchange is that of Linderstrøm-Lang (1955). According to the Linderstrøm-Lang hypothesis, exchange proceeds by a reversible transconformational reaction in which a nonexchanging or "closed" form, N, converts into an "open" or exchanging form, I,

$$\mathrm{N} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{I} \xrightarrow{k_3} \text{exchange} \qquad (3)$$

where k_1 is the rate constant for opening, k_2 is that for the closing reaction, and k_3 is the intrinsic rate constant of exchange for an unhindered peptide hydrogen. If the above model is assumed, it can be shown (Hvidt, 1964) that under conditions where $k_3 \ll k_2$, *i.e.*, low temperature and pH close to pH_{min}, the observed rate of exchange is given by

$$k_{obsd} = (k_1/k_2)k_3 = Kk_3 \qquad (4)$$

where K is the equilibrium constant for the opening reaction. Equation 4 then predicts that k_{obsd} (which is identical with the rate constants entered in Table I) should vary with pH as k_3 varies with pH, assuming that k_1 and k_2 are pH independent. Since the variation of k_3 with pH is known from Table II, we should find that

TABLE II: Comparison of First-Order Rate Constants for Poly-DL-alanine (k_3, min^{-1}) and Class III of Gramicidin S-A (k_{obsd}, min^{-1}).

pH	k_3 (Poly-DL-alanine)[a]	$k_{obsd}/k_3 = K$
5.0	2.49×10^{-1}	4.30×10^{-2}
4.7	1.25×10^{-1}	5.36×10^{-2}
4.0	2.49×10^{-2}	4.22×10^{-2}
3.7	1.86×10^{-2}	3.18×10^{-2}
3.0	7.16×10^{-3}	3.60×10^{-2}

[a] The k_3 values for pH 4.7, 3.7, and 3.0 are taken directly from Englander (1967). The k_3 values at pH 5.0 and 4.0 were calculated from the pH 4.7 data using eq 1.

$$k_{obsd}/k_3 = K, \text{ a constant} \quad (5)$$

The data in Table II show this to be the case for the very slow (class III) hydrogens of gramicidin S-A in the base-catalyzed region. Despite the fact that the observed rate of exchange of these hydrogens varies about 40-fold between pH 3.0 and 5.0, k_{obsd}/k_3 changes little in this region. While the available data allow few comparisons between the intermediate class (class II) in the base-catalyzed region with poly-DL-alanine, it should be noted that k_{obsd} changes linearly with hydroxyl ion concentration, in accordance with the known behavior of k_3. In addition, the rate of exchange of the four slow hydrogens in the acid-catalyzed region, between pH 2.4 and 2.0, changes linearly with hydrogen ion concentration, again in accordance with the known behavior of k_3. Thus, the above mechanism, often called the EX_2 or "bimolecular" mechanism, appears to hold for the slowly exchanging hydrogens of gramicidin S-A. It should be noted that most of the factors which complicate the interpretation of protein hydrogen exchange experiments are probably not present here. Gramicidin S-A contains only eight peptide hydrogens, all of which are observable under the proper conditions. The side-chain amino hydrogens of the ornithine groups have been shown, by nuclear magnetic resonance, to exchange too rapidly to be seen under our conditions (Stern *et al.*, 1968). It is difficult to imagine regions totally shielded from the solvent environment in a molecule of this size. Consequently, our data give substantial evidence for the validity of the EX_2 mechanism.[1]

From Table II, average values of K for the very slow class (class III) of hydrogens in gramicidin S-A may be calculated. Using the relation

$$\Delta G = -RT \ln K$$

where G is the Gibbs free energy, we may calculate the free-energy change associated with the $N \rightleftarrows I$ transconformational reaction for this class, in the base-catalyzed region. We may follow a similar procedure with the available data for the intermediate class in this region. The resulting values are 1.73 and 0.483 kcal per mole for these classes, respectively. Since the energy of this peptide has been estimated to be of the order of -100 kcal/mole (Scott *et al.*, 1967), it is clear that the conformational changes needed to allow the slow hydrogens to exchange are not major ones, and perhaps involve only small rotations about bonds.

The data in the acid-catalyzed region are more difficult to interpret. Comparison with the base-catalyzed region reveals that there are still four slower hydrogens. However, it is not clear why they are kinetically equivalent. Several explanations are possible: aggregation, a conformational change, crossing of pH–rate curves for two classes, steric effects, or charge effects. Molecular weight measurements with the analytical ultracentrifuge revealed no aggregation at pH 2.0. While the optical rotatory dispersion curve in the ultraviolet remained unchanged at low pH, the possibility of a small conformational change, perhaps arising through some protonation of the carbonyl oxygens, cannot be excluded. Indeed, thin film dialysis experiments show a definite decrease in the escape rate of gramicidin S-A on going from a pH of 3 to 2.4. The crossing of the pH–rate curve for two classes appears unlikely since there are four kinetically equivalent very slow hydrogens over at least 0.4 pH unit. In regard to the last two alternatives, it should be realized that the acid- and base-catalyzed reactions are known to proceed through different mechanisms (Berger *et al.*, 1959), the former having a positively charged and bulkier intermediate. There is no guarantee that steric effects, or charge effects involving the positively charged ornithine amino groups, would have identical effects on both reactions. This consideration is especially relevant in the light of new findings concerning the exchange of *N*-methylacetamide (Schleich *et al.*, 1968). In addition, ferrichrome A shows an increased number of very slow hydrogens in the acid-catalyzed region (Emery, 1967). If this phenomenon of changing kinetic class sizes is a general one, it may have relevance to protein hydrogen exchange studies. Thus, we cannot rigorously show that the slow hydrogens in the acid- and base-catalyzed regions are identical, although this appears likely in view of the conformational model discussed below. Since we have model compound data only for the base-catalyzed region, our attempts to relate exchange rate and structure of gramicidin S-A will focus on this region.

Owing to X-ray data obtained in the late 1950's (Hodgkin and Oughton, 1957), it is generally agreed that gramicidin S-A possesses a twofold axis of symmetry in the crystal. Recent nuclear magnetic resonance experiments carried out in this laboratory have shown that, in both methanol and dimethyl sulfoxide, identical amino acids in the molecule are found in equivalent environments, strongly suggesting a twofold axis of symmetry in these solvents (Stern *et al.*, 1968). Our tritium exchange data show that the kinetic class sizes are always approximately even numbers. This observation is consistent with the presence of a twofold axis in aqueous solution as well.

Proton transfer processes are known to be slowed by intramolecular hydrogen bonding (Eigen, 1964). This is due to the fact that a proton must hydrogen bond to an acceptor, in this case the solvent, before it may be transferred. Since proton transfers are part of the exchange process, exchange of hydrogen-bonded hydrogens in macromolecules such as DNA has been found to be slow (Printz and von Hippel, 1965, 1968). Another way in which exchange may be slowed is simply through inaccessibility to the solvent. In either case, the small size and cyclical nature of the gramicidin S-A molecule

[1] This conclusion may appear to contradict the observations of Emery (1967) for the cyclic peptide chelates ferrichromes A and B. The hydrogen exchange of these compounds was markedly insensitive to pH changes. However, this does not imply that the EX_2 mechanism is invalid. These forms may possess such low motility that $k_3 \gg k_2$ and k_1 becomes rate limiting. In this case k_{obsd} is expected to be relatively independent of pH, resulting in what Hvidt and Neilsen (1966) have termed an EX_1 or unimolecular exchange. EX_1 kinetics have recently been found for the crystalline form of insulin (Praissman and Rupley, 1968). Furthermore, the pH–rate data of Emery for another chelate, ferrioxamine B, strongly suggest the occurrence of an EX_2 mechanism.

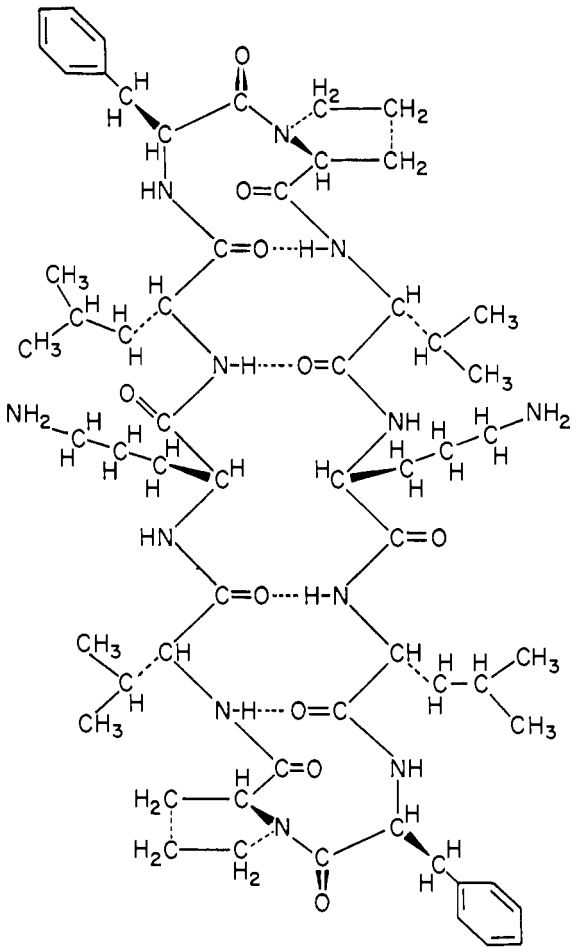

FIGURE 2: Schematic drawing of the model of Stern *et al.* for gramicidin S-A.

imply that the four slowly exchanging hydrogens must be located on the inside of the ring. The finding of two separate kinetic classes of slowly exchanging hydrogens in the base-catalyzed region indicates that the hydrogens of these two classes must be nonequivalent. Stern *et al.* (1968), using nuclear magnetic resonance, also found two classes of two slow amide hydrogens each in both methanol and dimethyl sulfoxide. The slowest were identified as those of valine and the next slowest as those of leucine. In the model proposed by these authors, shown in Figure 2, four hydrogen-bonded hydrogens are found. The bonds between the valine amide hydrogens and the leucine carbonyl oxygens are tucked in the extremities of the ring, while those between the leucine amide hydrogens and the valine carbonyl oxygens are found more to the center of the ring. In addition, the nitrogen–oxygen interatomic distance is considerably smaller for the former pair. From considerations of both steric effects and hydrogen-bond stability, the valine amide hydrogens would be expected to exchange more slowly than those of leucine. Thus, while our findings in the base-catalyzed region do not offer conclusive proof of hydrogen bonding, they are certainly consistent with the nuclear magnetic resonance data and with the above-mentioned model.

Several other aspects of our data may be explained with the aid of the proposed structure. It may be noticed that the four rapidly exchanging hydrogens, which Stern *et al.* have identified as the phenylalanine and ornithine amide hydrogens, appear to exchange somewhat faster than those of poly-DL-alanine in the base-catalyzed region. Owing to the method of analysis and the speed with which these hydrogens exchange, we have probably measured an average rate constant. Indeed, the nuclear magnetic resonance studies showed the phenylalanine amide hydrogens to exchange somewhat faster than those of ornithine. In model compound studies (Klotz and Feidelseit, 1966), it has been shown that *cis* amides exchange several times more rapidly than *trans* amides. In the proposed model for gramicidin S-A, the dihedral angle between the C=O and NH planes, ω, in the Leu–Phe peptide bond, is higher than the 0° associated with the *trans* peptide linkage. Since the bond is then somewhere between *trans* and *cis*, one would expect the phenylalanine peptide hydrogens to exchange faster than those of poly-DL-alanine, where all the linkages are *trans*. This would tend to increase the average rate constant observed for the four fast hydrogens above that of poly-DL-alanine at the same pH, as was found.

Another factor may contribute to the differences in exchange rate between the phenylalanine and ornithine amide hydrogens. Although both groups of hydrogens are located on the outside of the ring, the model shows those of ornithine to be partially buried beneath the amino group of the ornithine side chain. Viewed in terms of accessibility, this would tend to slow the exchange of the ornithine amide hydrogens. The presence of the positive charge on the side chain may shift the pH_{min} of these hydrogens to a lower pH, as well. Such a situation has been found for poly-L-lysine when positively charged (Leichtling and Klotz, 1966). The presence of a minimum below pH 3.0 could account for the relative insensitivity to hydrogen ion catalysis of two of the faster hydrogens, as shown in Table I.

It is important to note that our data for the hydrogen exchange of gramicidin S-A in dilute aqueous solution agrees quite well with the nuclear magnetic resonance data of Stern *et al.* in methanol and in dimethyl sulfoxide. This suggests that the conformations in these solvents are similar, if not identical, and that backbone energetics and steric constraints are therefore the prime conformational determinants for this molecule.

References

Balasubramanian, D. (1967), *J. Am. Chem. Soc. 89*, 5445.

Berger, A., Lowenstein, A., and Meiboom, S. (1959), *J. Am. Chem. Soc. 81*, 62.

Bryan, W. P., and Neilsen, S. O. (1960), *Biochim. Biophys. Acta 42*, 552.

Craig, L. C., Chen, H. C., Printz, M., and Taylor, W. I. (1968), *in* Characterization of Macromolecular Structure, McIntyre, D., Ed., Washington, D. C., Publication 1573 National Academy of Sciences, p 315.

Craig, L. C., Gregory, J. D., and Barry, G. T. (1950), *Cold Spring Harbor Symp. Quant. Biol. 14*, 24.

Craig, L. C., and Konigsberg, W. (1961), *J. Phys. Chem. 65*, 166.
Craig, L. C., and Stewart, K. (1965), *Biochemistry 4*, 2512.
Eigen, M. (1964), *Angew. Chem. Intern. Ed. Engl. 3*, 1.
Emery, T. (1967), *Biochemistry 6*, 3858.
Englander, S. W. (1963), *Biochemistry 2*, 798.
Englander, S. W. (1967), *in* Poly-α-amino Acids, Fasman, G. D., Ed., New York, N. Y., Marcel Dekker, p 339.
Englander, S. W., and Crowe, D. (1965), *Anal. Biochem. 2*, 579.
Hvidt, A. (1964), *Compt. Rend. Trav. Lab. Carlsburg, 34*, 299.
Hvidt, A., and Neilsen, S. O. (1966), *Advan. Protein Chem. 21*, 287.
Hodgkin, D. C., and Oughton, B. M. (1957), *Biochem. J. 65*, 752.
Klotz, I. M., and Feidelseit, P. L. (1966), *J. Am. Chem. Soc. 88*, 5103.
Klotz, I. M., and Frank, B. H. (1962), *Science 138*, 830.
Klotz, I. M., and Frank, B. H. (1964), *J. Am. Chem. Soc. 86*, 3889.
Leichtling, B. H., and Klotz, I. M. (1966), *Biochemistry 5*, 4026.
Linderstrøm-Lang, K. (1955), *Chem. Soc. 2*, 1.
Neilsen, S. O. (1960), *Biochim. Biophys. Acta 37*, 146.
Neilsen, S. O., Bryan, W. P., and Mikkelsen, K. (1960), *Biochim. Biophys. Acta 42*, 550.
Printz, M., and von Hippel, P. H. (1965), *Fed. Proc. 24*, 539.
Printz, M., and von Hippel, P. H. (1968), *Biochemistry 7*, 3194.
Praissman, M., and Rupley, J. A. (1968), *Biochemistry 7*, 2431.
Schleich, T., Gentzler, R., and von Hippel, P. H. (1968), *J. Am. Chem. Soc. 90*, 5954.
Scott, R. A., Vanderkooi, G., Tuttle, R. W., Shames, P. M., and Scheraga, H. A. (1967), *Proc. Natl. Acad. Sci. 58*, 2004.
Schwyzer, R., and Sieber, P. (1957), *Helv. Chim. Acta 15*, 624.
Segals, D. M., and Harrington, W. F. (1967), *Biochemistry 6*, 768.
Stern, A., Gibbons, W., and Craig, L. C. (1968), *Proc. Natl. Acad. Sci. 61*, 734.

Interaction of a Spin-Labeled Analog of Nicotinamide–Adenine Dinucleotide with Alcohol Dehydrogenase. I. Synthesis, Kinetics, and Electron Paramagnetic Resonance Studies*

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ABSTRACT: An analog of nicotinamide–adenine dinucleotide containing an unpaired electron has been prepared and shown to inhibit liver alcohol dehydrogenase competitively with respect to nicotinamide–adenine dinucleotide (K_I = 5 ± 2 μM). The compound, adenosine 5′-diphosphate-4(2,2,6,6,-tetramethylpiperidine-1-oxyl), was formed by coupling adenosine monophosphate to 2,2,6,6-tetramethyl-4-phosphopiperidine-1-oxyl. The electron paramagnetic resonance of the spin-labeled compound broadened when bound to liver alcohol dehydrogenase.

Titrating the enzyme with the spin-labeled analog and measuring the decrease in amplitude of the electron paramagnetic resonance spectrum revealed that the enzyme possessed two classes of binding sites: two sites which bind the analog with a K_D = 17 ± 8 μM, in agreement with its K_I, and five to six sites which bind with a K_D = 75 ± 9 μM. Reduced nicotinamide–adenine dinucleotide can only displace the bound radical from the strong binding sites, not from the five or six weak binding sites. Zinc-free liver alcohol dehydrogenase has been prepared and was shown to possess the two strong binding sites for the spin-labeled analog with a K_D = 27 ± 6 μM. The apoenzyme does not possess the weak binding sites. The analog can be displaced by reduced nicotinamide–adenine dinucleotide, showing that the enzymatically inactive apoenzyme can still bind coenzyme.

Fluorescence, absorption, and paramagnetic resonance spectroscopy have been used to study thermodynamic, kinetic, and structural properties of enzymes. The interactions of cofactors and enzymes have also been studied by these methods. For example, Boyer and Theorell (1956) have used fluorescence to study the binding of NADH to alcohol dehydrogenase (EC 1.1.1.1), and Beinert (Beinert *et al.*, 1962) used electron paramagnetic resonance to study flavoenzymes.

If the enzyme did not contain a group possessing the desired spectral properties, small molecules have been either chemically conjugated or bound to the enzyme in order to obtain the desired property. Enzymes conjugated to fluoroscein have been used for fluorescence studies (Steiner and Edelhoch, 1962), while "reporter" groups containing nitrophenol have been used for their absorp-

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