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Tritium–Hydrogen Exchange of Bacitracin A. Evidence for an Intramolecular Hydrogen Bond*

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ABSTRACT: Tritium–hydrogen-exchange kinetics of the polypeptide, bacitracin A, have been followed. The eleven peptide hydrogens, all of which appear on several exchange curves near the pH of minimum exchange rate, exchange as at least three distinct kinetic classes. Other potentially exchangeable hydrogens, such as free amino protons, the $C\alpha$ proton of the thiazoline ring, the C_2 proton of histidine, and probably the two primary amide protons, are not observed on the time scale of these experiments. The introduction of an additional negative charge by oxidation of the thiazoline sulfur of bacitracin A affects the exchange rate of all protons at all pH values studied. A single slow proton appears at all pH's and its exchange is less pH dependent than the rest of the peptide protons. This single hydrogen exchanges with a rate slower than that of poly-D,L-alanine. Cleavage of the peptide ring of oxidized bacitracin at the aspartic–asparagine bond produces a linear peptide which does not have slowly exchanging protons. Therefore, the single slow proton of bacitracin A may be participating in an intramolecular hydrogen bond within the peptide ring part of the molecule.

The exchange kinetics of the amide protons in polypeptides and polyamides are a sensitive probe of the secondary structure of these molecules (Hvidt and Nielsen, 1966). A slow amide exchange rate may indicate the presence of hydrogen bonding or inaccessibility of the amide proton to the solvent. For example, tritium–hydrogen-exchange (THX) experiments clearly indicated the presence of four hydrogen bonds in the secondary structure of the cyclic polypeptide antibiotic, gramicidin SA (Laiken *et al.*, 1969). In the cyclic peptide ferrichrome, four slowly exchanging protons have been found by the same technique. It was postulated that two of these arise from intramolecular hydrogen bonds while two are amide protons partially buried within the molecule (Emery, 1967).

The random coil structures of poly-D,L-alanine and poly-D,L-lysine have been examined by THX (Englander and Poulsen, 1969). Any transient hydrogen bonds that might exist in such a random coil polymer would not be expected to constrain the molecule to a unique secondary structure. Rather, such weak hydrogen bonds would form and break rapidly in different parts of the molecule due to the random thermal motions of the polymer chain. In these so-called random coil polypeptides, all amide protons apparently exchange at a similar rapid rate, since they are all in the same *average* environment. Evidence for a random coil structure with transient hydrogen bonds was found for poly-*N*-isopropylacrylamide by Scarpa *et al.* (1967). At zero degrees, hydrogens in peptides believed to be in random coil conformations exchange with a half-time of less than 100 min at the pH of minimum exchange rate. Under the same conditions, a proton in a hydrogen bond could be expected to exchange with a half-time of approximately 400 min or more. For example, α-helical poly-L-glutamic acid exchanges much more slowly than the random coil polymer, poly-L-lysine, at their respective pH_{min}'s (Leichtling and Klotz, 1966). Finally, oxidized ribonuclease, thought to be in a random coil, exchanges its amide protons at a very rapid rate compared to native ribonuclease, which has about one-hundred slowly exchanging hydrogens, whose rate is that expected for hydrogen-bonded protons (Englander, 1963). We find evidence for one slowly exchanging proton in the seven-residue peptide ring of the bacitracin molecule.

Bacitracin A is a product of a strain of *Bacillus licheniformis* and displays antibiotic activity against Gram-positive bacteria. Its structure, shown in Figure 1A, consists of a seven-membered peptide ring with a tail of five amino acid residues. The exchange study reported here was initiated for several reasons. The study of small peptides provides a bridge for testing the validity of extrapolating data from simple amides and homopolymers to proteins. Furthermore, if small peptides exist in a unique conformation characterized

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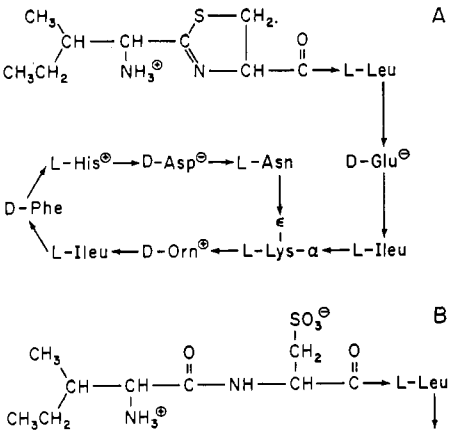

FIGURE 1: (A) Bacitracin A. Charges at pH 4.0 are shown. (B) Oxidized bacitracin. Only the amino-terminal residues are shown. The remainder of oxidized bacitracin is identical with bacitracin A.

by hydrogen bonding, they themselves may serve as models for structured proteins. Finally, knowledge of the secondary structure of antibiotic polypeptides may well be of value in understanding the nature of the interaction of the molecule with its receptor sites in bacterial and mammalian systems.

Experimental Section

Materials. Bacitracin A was isolated from a commercial preparation by countercurrent distribution (Craig *et al.*, 1969). All chemicals used were reagent grade and distilled water was deionized to ensure the absence of metal ions. Tritiated water (specific activity of one curie per milliliter) was obtained from New England Nuclear.

Oxidized bacitracin was prepared as described by Konigsberg *et al.* (1961). The linear bacitracin peptide was a gift from Dr. T. Hettinger. This derivative was prepared by mild acid hydrolysis of oxidized bacitracin and contains one less aspartic residue than does bacitracin A. Hydrolysis occurred at the *His–Asp* and *Asp–Asn* bonds.

Methods. Tritium–hydrogen-exchange experiments were performed by two methods. In one, rapid dialysis using a thin-film countercurrent dialyzer (Craig and Chen, 1969; Laiken *et al.*, 1969) was employed. Separation of the tritiated bulk solvent from the labeled peptide is achieved here by partition across a membrane which allows water to pass but passes only a small fraction of the peptide. The membranes were obtained by extensive acetylation of standard Visking dialysis tubing (Craig and Chen, 1969). The use of two thin films of liquid flowing countercurrently accelerates the dialysis process by orders of magnitude over static dialysis procedures. This method was used, however, where very fast exchange times were not important. The other technique, gel filtration (Englander, 1963), was employed both for fast exchange times and complete exchange curves. Thus we were able to compare the two techniques, and we established that they gave equivalent results.

Concentration determinations for bacitracin A were made by ninhydrin assay, or were determined spectroscopically at 252 or 225 nm on a Zeiss PMQII spectrophotometer. The oxidized and linear bacitracin peptides were determined by ninhydrin or at 225 nm. Extinction coefficients measured under experimental conditions were ϵ_{225} (bacitracin A) = 6250 M^{-1} cm^{-1}, ϵ_{252} (bacitracin A) = 2507 M^{-1} cm^{-1}, and ϵ_{225} (oxidized bacitracin) = 6518 M^{-1} cm^{-1}.

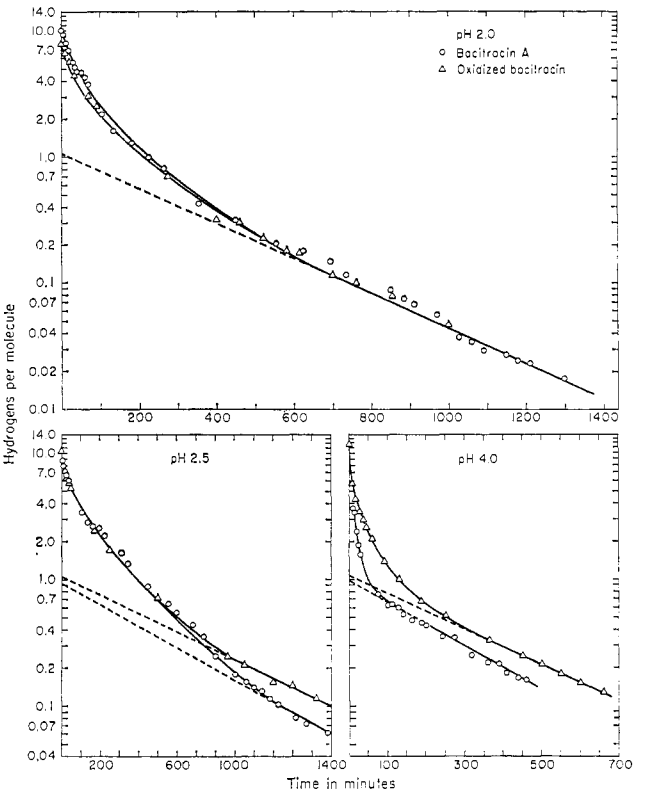

FIGURE 2: Tritium–hydrogen-exchange-out data at 0° for bacitracin A and oxidized bacitracin. Curves as pH 2.0 and 2.5 were obtained in 0.01 M phosphate and 0.1 M NaCl. The curve at pH 4.0 was in 0.01 M acetate and 0.1 M NaCl. The solid lines are computer drawn for bacitracin A. The dashed lines are extrapolations from the linear portions of the curves to zero time.

Experimental Procedure. The rates of exchange of the observable protons of bacitracin were defined by measuring the number of unexchanged hydrogens per mole of bacitracin as a function of time, after the exchange out of tritium had been initiated. Five to ten milligrams of bacitracin was dissolved in 250–1000 μl of buffer and equilibrated with a quantity of tritiated water to give a final specific activity of 10–20 mCi/ml. Equilibration was accomplished at room temperature and at the pH at which the exchange out was performed. During two to three hours of equilibration, at least five half-lives for the slowest hydrogen were realized. Twelve or more hours of equilibration caused the number of very slow hydrogens to fall consistently below one for reasons which are not known.

After equilibration, the sample was placed in ice, and the entire exchange process was performed at 0° by means of circulating ice water through jacketed columns within a specially constructed refrigerated box. At zero time the sample was pumped through a 90-cm countercurrent dialysis column or through a 5 × 0.9 cm column of Sephadex G-10, depending on the method being used. Passage through this first column separated the tritiated peptide from the bulk tritiated solvent. The peak tubes from this column were pooled and kept in ice. Aliquots of the pool were then passed through a second column, at recorded times, to remove tritiated bacitracin from the tritium which had exchanged out after passage through column one. Aliquots of the peak tubes from the second column were counted in Bray's solution in a Packard Tri-Carb liquid scintillation counter.

Since the exchange of tritium does not become unidirec-

TABLE I: First-Order Rate Constants, k_i (min^{-1}), and Class Sizes, C_i (Hydrogens per Molecule), for Bacitracin A at 0°.

pH	k_I	C_I	k_{II}	C_{II}	k_{III}	C_{III}
2.0	0.00308	0.99	0.0087	3.9	0.43	5.0[a]
2.5	0.00213	1.1	0.0044	3.9	0.017	3.0[a]
3.0	0.00239	0.92	0.0055	3.8	0.053	6.0
3.5	0.00243	0.94	0.014	3.8	0.017	6.2
4.0	0.00396	1.0	0.063	4.1		

[a] Remaining hydrogens in a faster class.

TABLE II: First-Order Rate Constants, k_i (min^{-1}), and Class Sizes, C_i (Hydrogens per Molecule), for Oxidized Bacitracin at 0°.

pH	k_I	C_I	k_{II}	C_{II}	k_{III}	C_{III}
2.0	0.00315	1.1	0.0107	4.0	0.0462	3.5[a]
2.5	0.00161	1.05	0.00577	4.2	0.0178	3.0[a]
3.0[b]	0.0013		0.0035			
3.5	0.00161	0.98	0.00660	3.9	0.0630	4.5[a]
4.0	0.00330	1.1	0.0198	3.8	0.077	4.0[a]

[a] Remaining hydrogens in a faster class. [b] From Figure 4 at pH_{min}.

tional at zero time, a time correction for the first column must be determined. This correction measures the elapsed time between injection of the sample and the point where tritium is no longer in equilibrium between the solvent and the peptide, but is effectively exchanging unidirectionally outward. The latter is assumed to occur when one order of magnitude of the original tritium in the bulk solvent is removed. Since the first column reduces the concentration of tritium in the bulk solvent by about six orders of magnitude, exchange out begins approximately one-sixth of the distance down the length of the column (Laiken *et al.*, 1969).

The number of hydrogens remaining per molecule after a given time of exchange was reproducible to within 5–10% by both the dialysis and gel filtration methods. The bulk of this error is due to contamination by extraneous tritium and to the difficulty of obtaining concentration measurements in dilute solution. The exchange curves presented are made up of points from at least two separate experiments.

Results

Figure 2 shows the number of unexchanged hydrogens remaining per molecule of bacitracin A and oxidized bacitracin as a function of time for three different pH values. At pH 2.0 and 2.5 the early time points approach the expected total of eleven exchangeable peptide protons. The long time portion of each curve is linear and extrapolates at each pH to one hydrogen at zero time. The bacitracin A curves were computer analyzed as described by Laiken and Printz (1970), in order to determine the number of statistically distinguishable classes of peptide hydrogens.

Briefly, the exchange can be characterized by eq 1 (Hvidt and Nielsen, 1966), where $H(t)$ is the number of protons per

$$H(t) = \sum_{i=1}^{n} C_i e^{-k_i t} \quad (1)$$

molecule remaining at time t. C_i is the number of hydrogens in the ith class and k_i is the observed rate constant for the ith class. Under our experimental conditions, k_{obsd} may be represented as the sum of three terms, one term for each possible mode of exchange. k_{H^+} and k_{OH^-} have dimensions

$$k_{obsd} = k_{H^+}[H^+] + k_{OH^-}[OH^-] + k_0 \quad (2)$$

of l. $mole^{-1}$ min^{-1} and are the catalytic rate constants for specific acid and specific base catalysis. k_0 has dimensions of min^{-1} and is the rate constant for a pH-independent exchange process.

Table I shows the observed rate constants for the slow class of one hydrogen, the intermediate class of four hydrogens, and the next fastest class of hydrogens of bacitracin A. The slowest class shall be designated C_I, the next class C_{II}, and the final group C_{III}. The observed rate constant for the slowest class shall be k_I, the next class k_{II} and so forth. Class III appears to contain at least three protons and possibly as many as all six remaining protons (seven for oxidized bacitracin), depending on the pH of exchange. It is difficult to obtain a statistically significant analysis of this region of the curve (less than 100 min of elapsed time). Table I shows that the number of hydrogens appearing in this region depends on the specific pH curve analyzed. The authors suggest that the six fast protons in bacitracin A exchange at rates which are not diverse enough to be consistently analyzed into distinctly separate classes by our present procedures. These protons may be under the influence of the carboxyl side chains of bacitracin, whose pK's occur within the pH region studied. Thus, the catalytic rate constants and the class sizes for class III and above might be pH dependent in the pH range 2.0–4.0. Therefore, Table I is interpreted as indicating at least one class of protons faster than class II. The numbers of hydrogens appearing in each class are computer generated and should be considered to be integral.

Table II gives the observed rate constants and class sizes determined for oxidized bacitracin. Note that the slow hydrogen is still present in oxidized bacitracin. This fact eliminated the possibility that the slow hydrogen is the C_α proton in the thiazoline ring in Figure 1A. If the C_α proton in bacitracin A were exchanging slowly due to its acidic character, it would not appear in oxidized bacitracin. Since the slow proton is still present in oxidized bacitracin, it is definitely a slow peptide proton.

Figure 3 shows a graphical analysis of the pH 4.0 exchange curve for oxidized bacitracin given in Figure 2. Figure 3A demonstrates analysis for the intermediate class of four protons, by subtracting the slow, one hydrogen class from the original curve. Figure 3B shows the appearance of class III of oxidized bacitracin.

Figure 4 presents a semilogrithmic plot of k_{obsd} as a function of pH for the class I and class II protons of oxidized bacitracin and bacitracin A. Table III summarizes the data presented in Figure 4. k_{H^+}, k_{OH^-}, and k_0 are the rate constants for the three possible modes of exchange calculated according to the method of Leichtling and Klotz (1966). The significance of Figure 4, Table III, and Table IV will be presented in the discussion.

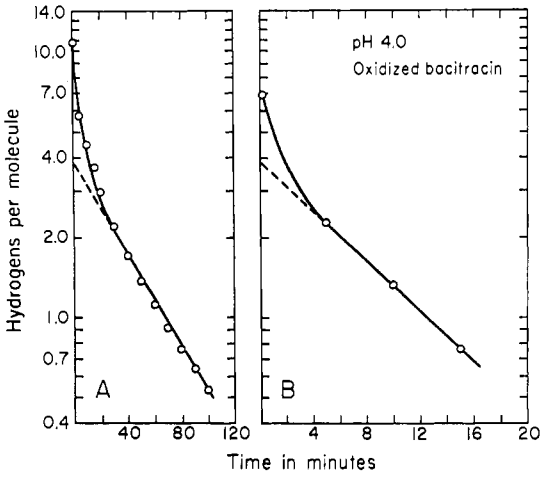

FIGURE 3: Graphical class analysis of the pH 4.0 exchange curve for oxidized bacitracin given in Figure 2. (A) Intermediate class of four hydrogens; the slow class of one hydrogen has been subtracted from the Figure 2 curve. (B) First fast class of oxidized bacitracin containing four hydrogens.

Figure 5 shows the exchange-out curve of the linear bacitracin peptide. The curve in Figure 5 should be compared to that of oxidized bacitracin in Figure 6, at 0.01 M NaCl. It is evident that linear bacitracin does not exhibit the one slow hydrogen present when the ring is intact. The net positive charge of linear bacitracin at pH 3.5 would be expected to accelerate the exchange rates of all protons at this pH, but probably not enough to cause class I to completely disappear.

The effect of ionic strength on the exchange kinetics of bacitracin A and oxidized bacitracin is shown in Figure 6. The exchange curves in 0.1 and 1.0 M NaCl are experimentally indistinguishable for oxidized bacitracin, which is electrophoretically neutral at this pH. However, bacitracin A appears to show a small salt effect in its fast class of hydrogens. There is no observed effect on the one slow proton.

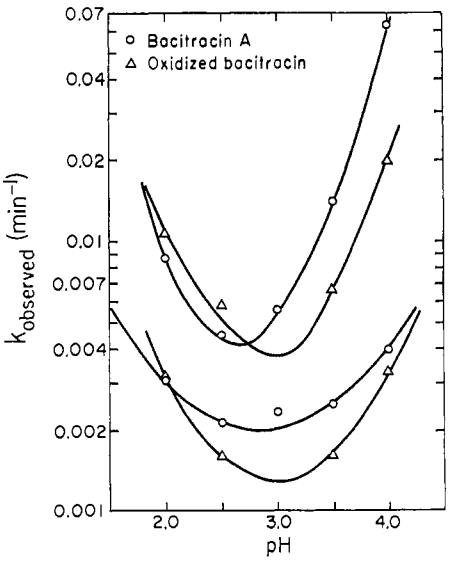

FIGURE 4: The observed rate constant as a function of pH for class I and class II of bacitracin A and oxidized bacitracin. The upper two curves are the four class II protons. The lower pair of curves are the class I protons.

TABLE III: Catalytic Rate Constants, k_{H^+} and k_{OH^-} (M^{-1} min^{-1}), k_0 (min^{-1}), and Minimum Rate Parameters, pH_{min}, and k_{min} (min^{-1}), for Bacitracin A and Oxidized Bacitracin at 0°.

	pH_{min}	k_{min}	k_{H^+}	k_{OH^-}	k_0
Bacitracin A					
Class I	2.85	0.0021	0.0034	1.59×10^8	0.0021
Class II	2.75	0.0041	0.22	5.5×10^9	0.0021
Oxidized bacitracin					
Class I	3.0	0.0013	0.25	2.5×10^8	0.0008
Class II	3.0	0.0037	1.33	1.33×10^9	0.0009

Since bacitracin A carries a net positive charge at pH 3.5, high salt concentration might be expected to shield peptide protons from this charge by supplying counterions. Thus, 1.0 M NaCl appears to slow the exchange of the fast bacitracin A protons in the base-catalyzed region. No significant salt effect was observed for either poly-L-glutamic acid (Leichtling and Klotz, 1966) or *N*-methylacetamide (Klotz and Frank, 1965).

Discussion

The structure of bacitracin A presented in Figure 1A is the result of recent data gathered in this laboratory. Previously proposed structures had located the asparagine outside the macrocyclic ring, attached to the aspartic (Hausmann *et al.*, 1955; Lockhart *et al.*, 1955). The more recent studies of Ressler and Kashelikar (1966) indicated, however, that it was in the ring, and unpublished data of Dr. T. Hettinger of this laboratory support their conclusion. Thus, bacitracin consists of a seven-membered ring with a tail of five residues.

Reactions a–c represent exchange as it occurs in the THX experiment where only the exchange of tritium is measured. Under the experimental conditions, no significant back-exchange occurs and the reaction effectively proceeds only from left to right. Specific base catalysis is a well-defined

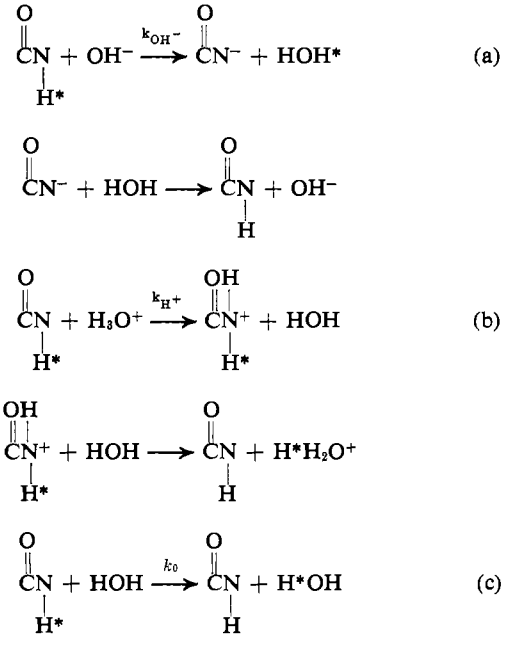

TABLE IV: Catalytic Rate Constants and Minimum Rate Parameters for Five Polypeptides at 0°.

Substance	Solvent, Method	pH_{min}	k_{min}	k_{H^+}	k_{OH^-}	k_0
Poly-D,L-alanine[a]	H_2O, THX	3.0	0.0085	3.5	3.5×10^9	0
Poly-D,L-lysine[a]	H_2O, THX	2.3	0.0036	0.087	8.8×10^9	0
Poly-L-glutamic acid[b]	D_2O–dioxane, infrared	3.20	3×10^{-6}	0.0035	1.5×10^7	1.2×10^{-6}
Poly-L-lysine[b]	D_2O–dioxane, infrared	2.55	0.0021	0.302	4.7×10^{11}	0
Gramicidin SA[c]	H_2O, THX					
Class I (two protons)		3.0	0.00025	0.122	1.22×10^8	0
Class II (two protons)		2.9	0.0018	0.124	1.93×10^8	0.0015
Class III (four protons)		2.8	0.0072	0.45	1.15×10^9	0.0058

[a] Englander and Poulsen (1969). [b] Leichtling and Klotz (1966). These data are corrected to 0° by the formula $(\partial \log k/\partial T)_{pH} = 0.05$ (Hvidt, 1964). Since the exchange occurred in D_2O, the data denote pD_{min}, etc. [c] Laiken *et al.* (1969).

process in which the amide proton is lost to a hydroxide ion in the rate-determining step. A proton is then acquired from water to complete the exchange. The process is described by reaction a and accounts for the second term in eq 2. Specific acid catalysis is less well defined since it is known that protonation of the amide carbonyl dominates protonation of the amide nitrogen (Berger *et al.*, 1959). Acid catalysis may be represented by reaction b and accounts for the first term in eq 2. The third term in eq 2, k_0, is the rate constant for a pH-independent exchange process. Although it is not known what pH-independent processes are important in the exchange of peptide protons, this mode of exchange might be that represented by reaction c. In general, exchange occurring through a pH independent mechanism should be significant only near pH_{min}. It is important near pH_{min} for class I of bacitracin A ($k_{min} = 0.0021$ min^{-1} and $k_0 = 0.0021$ min^{-1}) and for class II of gramicidin SA ($k_{min} = 0.0018$ min^{-1} and $k_0 = 0.0015$ min^{-1}). A pH-independent process is not observed for poly-D,L-alanine, poly-D,L-lysine, or poly-L-lysine.

In the overall exchange mechanism represented by eq 2, general acid and base catalysis have not been considered. It is possible that the pH-independent process which is observed for bacitracin and gramicidin SA is general acid or base catalysis by a functional group whose pK is far from the pH range studied or whose acid and base forms have identical catalytic rate constants. Experimental evidence for general catalysis of the exchange of amide protons has been presented by Leichtling and Klotz (1966).

The rates of reactions a and b are determined by steric or structural factors and by the charge environment of the amide if temperature and pH are maintained constant. Steric and structural effects always retard the exchange rate relative to that of an unhindered amide. This retardation is due to the partial inaccessibility of the amide to solvent. An amide proton may be partially inaccessible because it is buried within the structure of a large molecule, or because it is involved in an intramolecular hydrogen bond. We assume that bacitracin lacks both the large size and highly structured nature necessary to completely bury any peptide proton. The smaller peptide, ferrichrome, does contain a buried amide proton, but this is due to strong chelation of

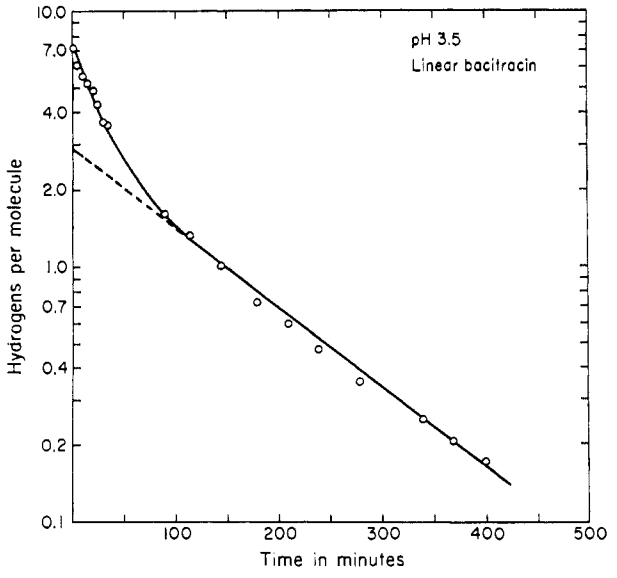

FIGURE 5: The exchange out at pH 3.5 of linear bacitracin, 0.01 M acetate, and 0.1 M NaCl. Note the time scale of this graph compared to that of Figure 6.

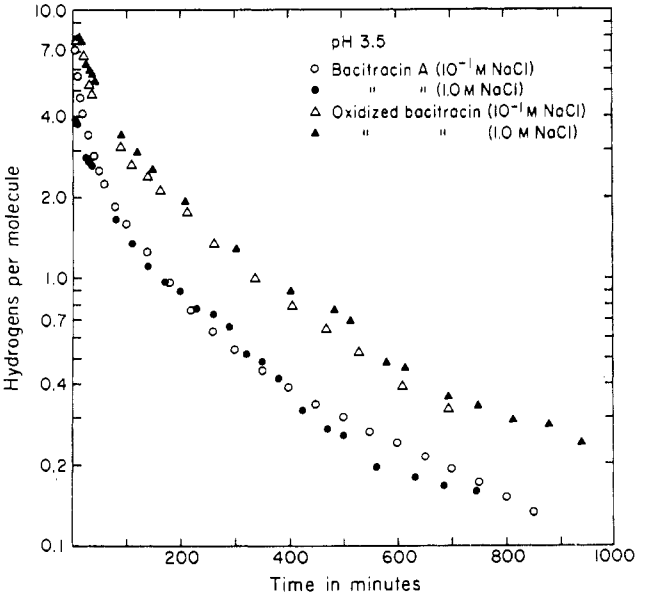

FIGURE 6: The exchange out of bacitracin A and oxidized bacitracin at pH 3.5 and varying ionic strength.

TABLE V: The Equilibrium Constant, K, for the Opening of the Hydrogen Bond in Oxidized Bacitracin.[a]

pH	k_3	$K = k_{obsd}/k_3$
2.0	0.036	0.088
2.5	0.012	0.13
3.5	0.012	0.13
4.0	0.036	0.092

[a] k_3 (min^{-1}) is the first-order rate constant for poly-D,L-alanine from Figure 5 of Englander and Poulsen (1969). k_{obsd} is from Table II of this paper.

ferric ion by the molecule which imposes a very rigid secondary structure (Emery, 1967). Thus, the class I proton is slow for one of two reasons. It may be involved in intramolecular hydrogen bonding, or else it may be the amide proton of the *Asn–ε-Lys* amide linkage. This amide linkage is not a normal α-peptide linkage. Its amide proton may be slowly exchanging due only to its location adjacent to the side chain of lysine instead of being adjacent to a continuation of the α-linked peptide backbone. The intrinsic exchange rate of the ε-lysine linkage in bacitracin has not yet been determined. The class II protons exchange at a rate similar to that of poly-D,L-alanine. The remaining protons exchange at a rate faster than poly-D,L-alanine for reasons which are not definitely known.

Kinetics. Both steric and charge effects have been treated theoretically by Leichtling and Klotz (1966). Experimentally, an amide proton which is sterically hindered in any way will exchange at a slower rate than a free amide. For a model unhindered amide without net charge, we may select poly-D,L-alanine. It has been shown that poly-D,L-alanine has $pH_{min} = 3.0$ and an exchange half-time of 82 min at 0°. A polyamide with larger side chains than poly-D,L-alanine will hinder the approach of a hydroxide ion in the base-catalyzed region and hydronium ion in the acid-catalyzed region with about equal efficiency. Therefore, the observed rate constant will decrease for all pH's but pH_{min} should remain the same. Class I of oxidized bacitracin exchanges with $pH_{min} = 3.0$ and a half-time of 530 min. Thus class I of bacitracin differs sterically from the protons of poly-D,L-alanine. Class II of oxidized bacitracin has $pH_{min} = 3.0$ and $t_{1/2} = 190$ min. Thus class II also differs sterically from poly-D,L-alanine protons, but it is not as hindered as class I. We assume that class II protons are probably not hydrogen bonded, due to their relatively rapid exchange, and to the difficulty of accomodating four more hydrogen bonds in the bacitracin ring, where such structure is most likely to occur.

The Linderstrøm-Lang model for sterically restricted hydrogen exchange assumes the following kinetic mechanism (Linderstrøm-Lang, 1955)

$$N \underset{k_2}{\overset{k_1}{\rightleftharpoons}} I \xrightarrow{k_3} \text{exchange}$$

The peptide is in equilibrium between two types of conformational states: N, the closed state, in which no exchange occurs, and I, the open state, in which exchange occurs at the rate of an unhindered peptide. This model is appropriate for the single slow hydrogen of bacitracin. For this proton, N is the hydrogen-bonded state. State I is the result of a small conformational change which transiently breaks the hydrogen bond, allowing exchange to occur. State I corresponds to an unhindered peptide with the same pH_{min} as bacitracin. In this case, poly-D,L-alanine is a good model for the I state of oxidized bacitracin. If $k_2 \gg k_3$ and $k_2 \gg k_1$, then k_{obsd} for the entire exchange process is (Hvidt and Nielsen, 1966)

$$k_{obsd} = \frac{k_1}{k_2} k_3 = Kk_3 \qquad (3)$$

The derivation assumes that the proton of interest spends most of its time in the N state and that state N is in equilibrium with a steady-state concentration of I.

The equilibrium constant for the opening process, K, may be calculated for oxidized bacitracin from eq 3, provided

$$K = \frac{k_{obsd}\ \text{(oxidized bacitracin)}}{k_3\ \text{(poly-D,L-alanine)}} = \text{a constant} \qquad (4)$$

k_1 and k_2 are pH independent. From Table V, $K = 0.11$ for class I of oxidized bacitracin. The relative invariance of K over the pH range 2.0–4.0 indicates that a pH invariant conformational change is occurring during which the hydrogen-bonded state N opens to the freely exchangeable state I. From the relation $\Delta G° = -RT \ln K$, the free-energy change for the opening process is 1.2 kcal/mole. The free-energy changes associated with the opening of the hydrogen bonds in gramicidin SA are 1.73 and 0.483 kcal per mole, respectively.

Unlike steric hindrance, net charge does not equally affect the approach of a hydroxide and a hydronium ion. Net positive charge will repel hydronium ion and retard acid-catalyzed exchange, while enhancing the approach of hydroxide ion and accelerating base-catalyzed exchange. Net negative charge has the opposite effect. The net positive charge of poly-D,L-lysine therefore accelerates base-catalyzed exchange and retards acid-catalyzed exchange compared to poly-D,L-alanine (both polymers are random coil). From Table IV, k_{OH^-} is increased in going from poly-D,L-alanine to poly-D,L-lysine and k_{H^+} is decreased. The net effect is to shift pH_{min} toward lower pH without having much effect on k_{min}. From Table IV, $[H^+]_{min}$ changes by a factor of about 5 from the neutral to the positively charged polymer. k_{min} is changed by a factor of less than three.

The effect of net charge environment on exchange kinetics has been strikingly demonstrated by adding the anionic detergent sodium dodecyl sulfate to neutral polyisopropylacrylamide and observing the exchange in D_2O (Klotz and Mueller, 1969). pD_{min} for the polyamide alone is 5.0 with a half-time of 690 min at 15°. In the presence of the detergent, which binds to the polymer, pD_{min} is shifted to 6.5 with $t_{1/2} =$ 230 min.

In Table III the effect of net molecular charge is apparent in comparing oxidized bacitracin to bacitracin A, the latter having one less negative charge than the former. For the protons in class II, the $[H^+]_{min}$ changes by a factor of 2 in going from oxidized bacitracin to bactracin A while the half-time of these protons changes by only about 10%. This effect may be seen pictorially in Figure 4. As is expected, k_{H^+} is larger for the bactracin with the additional negative charge, 0.25 M^{-1} min^{-1} compared to 0.0034 M^{-1} min^{-1}, and k_{OH^-} is smaller, 1.33×10^9 M^{-1} min^{-1} compared to

5.5×10^9 M^{-1} min^{-1}. Similar results, due to a difference in charge apply to the class I protons. The relevant data are in Table III.

It might have been expected that charged side chains, such as those present in bacitracin, would strongly influence the exchange of the nearest peptide proton without significantly affecting the other protons. Thus, the two peptide protons immediately adjacent to the glutamic residue might exchange at a higher rate in the acid-catalyzed region than protons near neutral residues. Likewise, the two protons adjacent to the positively charged ornithine residue might exchange more rapidly in the base-catalyzed region and less rapidly in the acid-catalyzed region than protons near neutral residues. By using such correlations, the classes of bacitracin might have been assignable to specific protons according to the proximity of certain protons to charged side chains. However, class I and class II exchange with very nearly the same pH_{min} in oxidized bacitracin and bacitracin A, respectively. Thus the five protons in these two classes see the same effective charge or inductive effect within each bacitracin molecule and differ in rate due to a steric factor.

The introduction of a negative charge by oxidizing bacitracin A does not cause the appearance of an additional class II or class I proton on the base-catalyzed side of pH_{min}, nor does it markedly change the rate of any individual proton alone. This negative charge appears to affect *all* measurable classes of protons, primarily by shifting pH_{min}. The localized effect of a charged side chain on nearest neighbor protons may be present in class III hydrogens. However, this class is not well enough resolved in this study to show large charge effects on specific protons. It is therefore *net* molecular charge which appears to affect exchange in this study and not the precise location and number of individual positive and negative charges. This effect may be characteristic of molecules of this size bearing several charged groups. However, in small molecules such as triglycine, the base-catalyzed exchange rate of the proton nearest the positive amino terminus is nearly two orders of magnitude faster than the proton nearest the carboxy terminus (Scheinblatt, 1966, 1970). The magnitude of the effect is much smaller when the carboxyl group is not charged. Similar effects of specific side chains on specific protons might appear in a molecule with a simpler charge distribution than bacitracin. Such a molecule with one positive charge well separated from one negative charge might be expected to exchange in a manner more like triglycine than bacitracin.

Evidence for a Hydrogen Bond. The primary evidence for internal hydrogen bonding in bacitracin is the abnormally slow exchange rate of a single peptide hydrogen. It has previously been shown that internal hydrogen bonding retards diffusion-controlled proton transfer by one or more orders of magnitude (Rose and Stuehr, 1968). Polypeptides which are known to possess secondary structure exhibit a correspondingly slow rate of exchange for the protons involved in hydrogen bonds.

Table IV provides several dramatic examples of the effect of hydrogen bonding on the exchange of peptide protons. In D_2O–dioxane the half-time of poly-L-glutamic acid is 2.3×10^5 min at pD_{min} while that of poly-L-lysine is 330 min at pD_{min}. Poly-L-glutamic acid is believed to be helical and poly-L-lysine is believed to be a random coil under these conditions. Gramicidin SA is known to have two pairs of hydrogen bonds from evidence independent of exchange data (Stern *et al.*, 1968; Ovchinnikov, 1970). The slowest pair, class I, exchanges with a half-time of 2780 min at $pH_{min} = 3.0$. The other pair, class II, has $t_{1/2} = 385$ min at $pH_{min} = 2.9$. This is a large retardation in rate compared to the random coil protons of poly-D,L-alanine with a half-time of 82 min at $pH_{min} = 3.0$. The nonhydrogen-bonded protons of gramicidin, class III, have a half-time of 96 min at pH_{min}, in good agreement with the nonhydrogen-bonded protons of poly-D,L-alanine.

The slow proton of oxidized bacitracin at $pH_{min} = 3.0$ has $t_{1/2} = 530$ min. The only other polypeptides for which there are comparable exchange data are poly-D,L-alanine and gramicidin SA. (In order for exchange data to be comparable, exchange must occur in relatively dilute aqueous solution, and the pH_{min} must be similar.) With $pH_{min} = 3.0$, poly-D,L-alanine protons have $t_{1/2} = 82$ min and class II of gramicidin SA has $t_{1/2} = 385$ min. The slow proton of bacitracin is thus probably hydrogen bonded. This slow proton is located in the ring by the experimental curve in Figure 5. When the seven-membered ring of bacitracin is cleaved, the slow proton is no longer present.

Figure 5 also provides evidence that the slow proton of bacitracin is not the amide proton of the *Asn–ε-Lys* linkage, which may be an intrinsically slowly exchanging site. If the slow proton were the *ε-Lys* amide, it should also be slow in the linear bacitracin peptide, and Figure 5 shows that it is not. Of course, this amide proton could be the slow proton due to its involvement in a hydrogen bond in the intact ring.

The Conformation of Bacitracin. Few details of the secondary structure of bacitracin A are known. Thin film dialysis studies in this laboratory indicate a compact configuration with the tail folded over the ring. Metal binding studies (Cornell and Guiney, 1970; Craig *et al.*, 1969; Garbutt *et al.*, 1961) implicate the α-amino terminus, the histidine, and the thiazoline nitrogen as ligands for zinc(II) and copper(II). In order to chelate metals with the proposed ligands, the amino terminus must be near the histidine. Since dialysis studies indicate no change in diffusional size upon binding zinc, the tail is therefore compactly folded over the ring both with and without the presence of the metal.

This exchange study adds one more potential structural feature to the bacitracin molecule. If the *ε-Lys* amide proton is not intrinsically slowly exchanging, then there is one intramolecular hydrogen bond in the seven-residue ring. If this bond is present, the authors suggest that it may be present in a β turn at one end of the ring. The β turn has been suggested by Urry *et al.* (1970) for the cyclic moiety of oxytocin, and consists of a hydrogen bond between the carbonyl of residue i and the peptide proton of residue $i + 3$.

Another possibility is a hydrogen bond involving the *ε-Lys* amide proton and the *Orn* carbonyl, with the alkyllysyl side chain "turning a corner" (not necessarily in the plane of the ring) to form one end of the ring. Either of these possibilities present the likelihood that the ring forms an anti-parallel conformation similar to that found for gramicidin SA (Stern *et al.*, 1968), but without extensive hydrogen bonding. These two possibilities seem likely only because they allow relatively compact folding of the tail over the ring with Corey–Pauling–Koltun space-filling models. All other singly hydrogen-bonded ring conformations cannot be ruled out provided they allow a folding which permits the α-amino terminus and the thiazoline nitrogen to be in position to chelate a metal ion with histidine.

In summary, bacitracin clearly illustrates several known characteristics of exchangeable peptide hydrogens. All peptide protons (11 for bacitracin A and 12 for oxidized bacitracin) appear on exchange curves near pH_{min}. These

protons exchange with rates which are influenced by the net molecular charge of bacitracin and the steric environment of each individual proton. Oxidized bacitracin exchanges with the same pH_{min} as poly-D,L-alanine, but bacitracin A has one less negative charge than oxidized bacitracin, and thus exchanges with a lower pH_{min}. Both bacitracins show the same slow and intermediate classes of hydrogens. The abnormally slow proton in bacitracin is either in a single intramolecular hydrogen bond in the macrocyclic ring or is the ϵ-*Lys* amide proton, which may be intrinsically slowly exchanging. If a hydrogen bond is present, it must allow a secondary structure with the tail compactly folded over the ring.

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Reversible Pressure–Temperature Denaturation of Chymotrypsinogen*

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ABSTRACT: The pressure–temperature-reversible transition surface for chymotrypsinogen at pH 2.07 has been determined by ultraviolet difference spectra over the temperature interval 8.5 to 70° between atmospheric pressure and 7000 atm. Assuming the transition involves two states, it is shown that the surface can be fit to a relatively simple equation of state which elicits approximately elliptical contours of constant free-energy difference on the pressure–temperature plane. Similar results are obtained by analyzing the recent ribonuclease data of Brandts *et al.* (*Biochemistry 4*, 1038 (1970)), and in both cases the equation of state is found to be compatible with known denaturation phenomena associated with pressure–temperature interactions, including the cold melting phenomenon. Values are calculated for the apparent thermodynamic transition parameters, *i.e.*, $\Delta\alpha$, $\Delta\beta$, ΔC_p, ΔS, ΔV, and ΔG.

In 1914 Bridgman observed that elevated hydrostatic pressure irreversibly denatures egg albumin. Since that time a number of investigations have revealed that pressure denaturation of proteins exhibits some unusual and interesting characteristics. It has been observed, for example, that while very high pressures (7500 atm) invariably produce protein denaturation, moderate pressures (1000 atm) may stabilize the native form, thereby increasing the temperature

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