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Tritium–Hydrogen Exchange of the Cyclic Peptide Polymyxin B_1†

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ABSTRACT: The exchange kinetics of the amide hydrogens of the basic polypeptide antibiotic polymyxin B_1 have been measured by a tritium–hydrogen exchange (THX) technique. The 11 amide hydrogens of polymyxin B_1 are observed near the pH of minimum exchange rate, while fewer hydrogens are observed away from pH_{min}. At all pH values studied, one amide proton exchanges significantly more slowly than the others. A single more slowly exchanging amide proton is also observed in a noncyclic heptapeptide derived from the ring portion of polymyxin B_1. However, comparison of the observed rate constants for specific acid and base catalyzed exchange in polymyxin B_1 with the theoretically calculated rate constants shows that in spite of one proton exchanging more slowly than the others no amide protons are involved in internal hydrogen bonding. Comparison of the exchange rates of the two slowest classes of amide protons of polymyxin B_1 and the acyclic ring peptide of polymyxin B_1 confirms an earlier conclusion that the cyclic portion of polymyxin B_1 is not stabilized by internal hydrogen bonding.

There now seems good reason to believe that the biological activity of many small natural polypeptides may be related to their solution conformations which are in turn determined by elements of secondary structure, *i.e.*, internal hydrogen bonding, hydrophobic interactions, electrostatic interactions, solvent effects, etc. The presence of strong hydrogen bonds places major restrictions on the number of conformational forms which need be considered for the polypeptide and thereby greatly simplifies conformational analysis. The exchange kinetics of the amide hydrogens of small polypeptides have proven to be sensitive probes of the secondary structure of these molecules (Laiken *et al.*, 1969; Galardy *et al.*, 1971; Printz *et al.*, 1972b). An amide proton exchanging sufficiently slowly under conditions of both specific acid and specific base catalysis probably indicates the presence of an intramolecular hydrogen bond or steric hindrance to access to the solvent in a small peptide.

The clearest example of such a relationship is found in gramicidin SA, a cyclic decapeptide. Our THX[1] data indicated the presence of four intramolecular hydrogen bonds in gramicidin SA (Laiken *et al.*, 1969) in agreement with the nuclear magnetic resonance (nmr) observations and the proposed model for gramicidin SA (Stern *et al.*, 1968). A single slowly exchanging amide proton was also found by THX in the cyclic peptide antibiotic bacitracin A (Galardy *et al.*, 1971). This amide proton is thought to be involved in an intramolecular hydrogen bond due to its slow exchange rate in native bacitracin A and its fast exchange rate in a bacitracin peptide with the cyclic portion of the molecule cleaved. THX of angiotensin II in aqueous solution containing 0.1 M NaCl showed two slowly exchanging amide protons which suggested two intramolecular hydrogen bonds (Printz *et al.*, 1972b). On the basis of the THX results and nmr data (Bleich *et al.*, 1973a,b), a solution conformation was proposed for angiotensin II (Printz *et al.*, 1972a) which was also found to be stable by potential energy calculations (Nemethy and Printz, 1972).

Although the cyclic portion of polymyxin B_1 (see Figure 1a) is topologically similar to the cyclic portion of bacitracin A, the results reported here suggest that, in contrast to bacitracin A, polymyxin B_1 contains no strong intramolecular hydrogen bonds. This lack of specific secondary structure is consistent with the nonspecific, detergent-like, antibacterial activity originally associated with the polymyxins (Goodman and Gilman, 1970). A solution conformation for polymyxin B_1 without internal hydrogen bonding is also consistent with the recently proposed mechanism of polymyxin B action based on the specific interaction between polymyxin B and certain phospholipids (Hsu Chen and Feingold, 1973).

Experimental Section

Materials. The structure of polymyxin B_1 is given in Figure 1 (I). The "B" refers to the presence of D-Phe in the variably substituted position in the ring of the polymyxin antibiotics. The subscript "1" refers to the particular fatty acid attached to the α-amino terminus, 3-methyloctanoic acid. The polymyxin antibiotics are products of *Bacillus polymyxa* and are bacteriostatic and bacteriocidal agents for a wide variety of gram-negative organisms. The peptide shown in Figure 1

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[1] Abbreviations used are: THX, tritium–hydrogen exchange; Dab, α,γ-diaminobutyric acid; pH_{min}, the pH of the minimum exchange rate; $t_{\frac{1}{2},min}$, the half-time for exchange at pH_{min}.

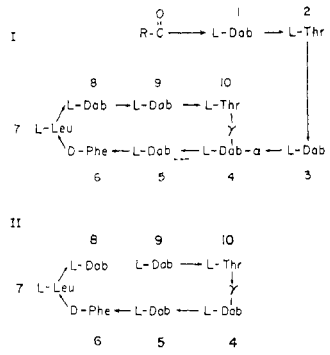

FIGURE 1: (I) The covalent structure of polymyxin B_1 where RCOOH is 6-methyloctanoic acid. (II) The covalent structure of the acyclic polymyxin B_1 ring peptide.

(II) was isolated from a Subtilopeptidase A digestion of a commercial mixture of polymyxin B_1 and B_2 (Pfizer) and is derived from the ring portion of these polymyxins. This peptide will be referred to as the polymyxin B_1 ring peptide. The purification of polymyxin B_1 and the preparation and characterization of the polymyxin B_1 ring peptide will be reported elsewhere (R. E. Galardy *et al.*, submitted for publication).

Distilled water was used for all buffer solutions and all chemicals were reagent grade. Tritiated water with a specific activity of 1 Ci/ml was obtained from New England Nuclear Corp.

Methods. The Sephadex gel filtration technique was used for all exchange curves (Englander, 1963; Printz, 1970). A brief discussion of the technique as applied to polypeptides and the general method for determining time corrections has been presented (Galardy *et al.*, 1971). The experiments utilized both the single and double pass systems (Printz, 1970), the former for exchange times of less than 3 min. The procedure for the single pass experiments was as follows: 2–3 mg of peptide was dissolved in 150 μl of buffer. Tritiated water was added to a final specific activity of 3–6 Ci/l. and the solution was incubated at room temperature for a time sufficient to realize at least 10 half-lives of exchange-in for the slowest hydrogen, usually 18 hr, in the buffer to be used for the exchange-out. To initiate an experiment, 20 μl of this solution was injected into the Sephadex column and eluted at a flow rate of approximately 1.5 ml/min. By applying the appropriate time correction, the number of unexchanged hydrogens remaining per molecule upon elution could be determined. The shortest time we obtained was 38 sec of exchange-out. Times up to 3 min were obtained by stopping the buffer flow when the peptide band was approximately halfway down the column for appropriate time intervals, and then continuing elution in order to separate the tritium which had exchanged out of the peptide during the stopped time on the column. The procedure for the double pass experiments has been described (Galardy *et al.*, 1971).

The final concentrations of the tritiated peptides were determined spectroscopically at 225 nm on a Zeiss PMQII spectrophotometer. Extinction coefficients for the peptides were: $\epsilon_{225,\ \text{polymyxin B}_1}$ 2490 and $\epsilon_{225,\ \text{pylymyxin B}_1\ \text{ring peptide}}$ 1970. The specific activities of the tritiated peptides were determined by scintillation counting in 10 ml of Bray's solution in a Packard TriCarb liquid scintillation counter. Samples were counted to 10,000 counts in order to realize a probable counting error of 0.6%. The number of hydrogens per molecule remaining after a given time of exchange-out was reproducible to within 5% by both the single and double pass methods for >0.1 hydrogen remaining. The bulk of this error was probably due to contamination by extraneous tritium and to the error in absorbance measurements on dilute solutions.

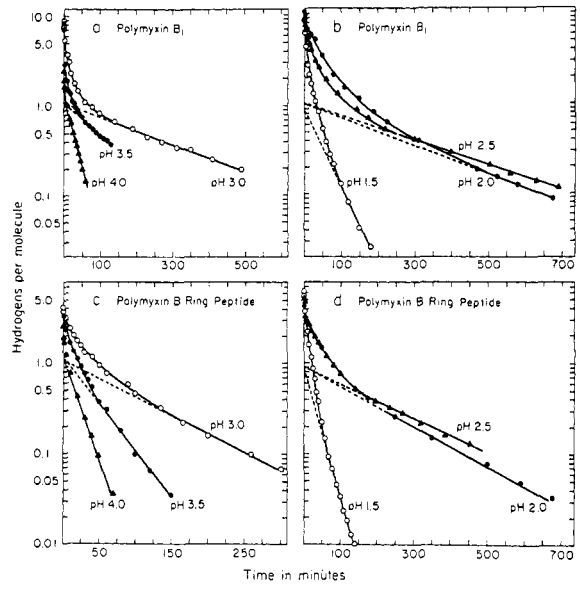

FIGURE 2: Exchange-out curves for polymyxin B_1 and the polymyxin B_1 ring peptide at 0°. The buffers used were as follows: pH 4.0 0.01 M NaOAc–0.1 M NaCl; pH 3.5 0.01 M sodium formate–0.1 M NaCl; pH 3.0 0.01 M sodium formate–0.1 M NaCl; pH 2.5 0.01 M NaH_2PO_4–0.1 M NaCl; pH 2.0 0.01 M NaH_2PO_4–0.1 M NaCl; pH 1.5 0.1 M H_3PO_4–0.07 M NaCl.

Results

The exchange-out can be characterized by

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

where $H(t)$ is the number of unexchanged hydrogens remaining per molecule after t minutes of exchange-out, C_i is the number of hydrogens in the ith class characterized by the observed rate constant k_i, and n is the total number of classes of exchangeable hydrogens observed. The assignment of exchanging amide hydrogens to classes is a convenience due to the impossibility of extracting the exchange rate of each individual hydrogen from $H(t)$ (Laiken and Printz, 1970). Our analysis of polymyxin B_1 distinguishes three classes: the slowest class defined by the terminal portion of the exchange-out curve, the next slowest class obtained by graphically subtracting the slowest class from the original exchange-out curve, and the fastest class consisting of the remaining hydrogens.

Under our experimental conditions (*i.e.*, near pH_{min} and with low concentrations of buffer acids and bases) k_i may be represented as the sum of three terms, one for each possible mode of exchange: $k_i = k_H(H^+) + k_{OH}(OH^-) + k_0$. k_H and k_{OH} have dimensions of M^{-1} min^{-1} and are the 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. k_0 is small for polymyxin B_1 and for the ring peptide.

The exchange-out curves for polymyxin B_1 and the polymyxin B_1 ring peptide are shown in Figure 2 for pH's near pH_{min}. In b, extrapolation of the exchange-out curve for polymyxin B_1 at pH 2.0 approaches 11 hydrogens per mol-

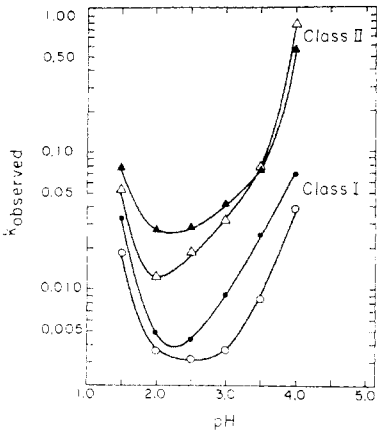

FIGURE 3: The pH–rate profiles of the two slowest classes of amide protons of polymyxin B_1 (○,△) and the polymyxin B_1 ring peptide (●,▲) at 0°. k_{obsd} in M^{-1} min^{-1}.

ecule at zero time, the correct number of amide hydrogens in the molecule. In d, the exchange-out curve for the polymyxin B_1 ring peptide at pH 1.5 shows about six hydrogens per molecule at zero time, the correct number of amide hydrogens in the molecule. Thus, all of the amide protons are apparently being observed in these THX experiments. For each exchange-out in Figure 2 the long time portion of the curve is linear and extrapolates to one hydrogen at zero time, defining the slowest exchanging class (class I). The observed half-times of exchange for class I and class II and the number of protons found in each class for polymyxin B_1 and the ring peptide are given in Table I.

Figure 3 shows k_{obsd} *vs.* pH for class I and class II of polymyxin B_1 and the polymyxin B_1 ring peptide. Table II gives the parameters of the minimum exchange rate and the rate constants for specific acid and specific base catalyzed exchange for these classes of the two peptides, and of poly-D,L-Ala and poly-D,L-Lys (Englander and Poulsen, 1969). k_H and k_{OH} for class I were calculated according to the method of Leichtling and Klotz (1966). For class II, k_H and k_{OH} were estimated from the extremes of the pH rate profiles in Figure 3.

Table III shows the rate constants for specific acid and specific base catalyzed exchange of the amide protons of polymyxin B_1 and the ring peptide calculated by the method of Molday *et al.* (1972). The numbering of the amino acid residues is shown in Figure 1. The relative rate corrections, $\log k_H^R - \log k_H^{Ala}$ and $\log k_{OH}^R - \log k_{OH}^{Ala}$, were calculated for Dab-1, -3, -5, -8, and -9 by using the values given for Lys. The Dab^4-γ-amide proton in polymyxin B_1 was considered to be equivalent to the methylamide proton of the model compound N^α-acetyllysine methylamide and the relative rate correction for this amide proton was estimated using data given by Molday *et al.* (1972). The Dab^4-α-amide proton was considered to be equivalent to lysine α-amide proton of N^α-acetylalanyllysine methylamide and the Dab^1-amide proton to the lysine α-amide proton in N^α-acetyllysine methylamide. The Dab$^{4\text{-}1}$-amide proton in the polymyxin B_1 ring peptide was considered to be equivalent to the methylamide proton of the model compound lysine methylamide and the relative rate correction for this amide proton was estimated using data given by Molday *et al.* (1972). The relative rate corrections for the remaining amide protons were taken directly from Table IV of Molday *et al.* (1972).

TABLE I: Half-Times of Exchange in Minutes ($t_{1/2}$) and the Number of Protons Found in Each Class (C_i) for the Two Slowest Classes of Amide Protons in Polymyxin B_1 and the Polymyxin B_1 Ring Peptide at 0°.

	Polymyxin B_1				Polymyxin B_1 Ring Peptide			
pH	$t_{1/2,I}$	C_I	$t_{1/2,II}$	C_{II}	$t_{1/2,I}$	C_I	$t_{1/2,II}$	C_{II}
4.0	18	1.2	0.8	1.9	10	1.2	1.3	2.1
3.5	81	1.1	9	2.0	28	1.2	9.5	1.6
3.0	195	1.1	22	1.5	75	1.1	14	1.8
2.5	215	1.0	38	2.8	160	0.9	25	2.3
2.0	195	1.0	57	3.0	155	0.8	24	3.3
1.5	38	0.9	13	3.0	21	0.9	9	3.8

Discussion

Comparison of k_H and k_{OH} found for classes I and II of polymyxin B_1 and the ring peptide show that the observed hydrogens have similar exchange rates in both peptides (Table II). This indicates that in aqueous solution the intact ring of polymyxin B_1 does not possess an internally hydrogen bonded structure since this structure would probably be broken in the noncyclic ring peptide and thus lead to grossly different exchange rates than in the ring peptide. The calculated exchange rates in Table III also indicate that in aqueous solution there are no internally hydrogen bonded amide protons of significant strength in either the ring peptide or in polymyxin B_1 since the observed exchange rates are all equal to or faster than the calculated rates. (See the data in Printz *et al.* (1972b) for a good comparison.) From these comparisons it is clear that polymyxin B_1 has no strong internal hydrogen bonds. This conclusion is in partial disagreement with the conclusions from nmr data of two other laboratories (Chapman and Golden, 1972; Urry and Ohnishi, 1970). The conclusion of Urry and Ohnishi was based on the observation that chemical shifts of two of the protons did not change with a change in temperature as much as the others even though a considerable shift was noted. This is now known not to be a reliable indication of hydrogen bonding. Neither laboratory based their conclusions on results at the pH minimum of exchange. Chapman and Golden apparently based their results on experiments in Me_2SO. When they dissolved the polymyxin in deuterium oxide the exchange was so rapid they could not measure it.

A quantitative comparison of the calculated exchange rate constants in Table III with the observed exchange rate constants in Table II shows poor overall agreement, however. The best agreement is observed for k_H in polymyxin B_1. If Dab^1 and Dab$^{4\text{-}1}$ are in the fastest exchanging class of polymyxin B_1, then the calculated k_H's for the remaining protons agree well with the observed k_H's for class I and class II, although one class I and two or three class II protons are not indicated from the calculated rate constants. The agreement between calculated and observed k_{OH} for polymyxin B_1 is similar to that found for k_H. For the polymyxin B_1 ring peptide, the agreement between calculated and observed k_H and k_{OH} is very poor. There appears to be no obvious explanation for these discrepancies, especially since measurement of k_{OH} for the polymyxin B_1 ring peptide in this laboratory by an nmr technique at alkaline pH gives excellent agreement with the calculated k_{OH} (R. Bockman, unpublished data). A possible explanation for the discrepancy between the THX and the

TABLE II: Parameters of the Minimum Exchange Rate and the Rate Constants for Specific Acid and Base Catalysis for Polymyxin B_1, the Polymyxin B_1 Ring Peptide, Poly-D,L-alanine (Englander and Poulsen, 1969), and Poly-D,L-lysine (Englander and Poulsen, 1969) at 0°.

Peptide	Class i	C_i[a]	pH_{min}	$t_{1/2min}$ (min)	k_H (M^{-1} min^{-1})	k_{OH} (M^{-1} min^{-1})
Polymyxin B_1	I	1	2.5	220	0.3	3.0×10^9
	II	2–3	2.0	58	1.7	2.1×10^{10}
Polymyxin B_1 ring peptide	I	1	2.2	190	1.0	7.8×10^9
	II	2–4	2.2	28	2.4	2.0×10^{10}
Poly-D,L-Ala	I	All	3.0	82	3.5	3.5×10^9
Poly-D,L-Lys	I	All	2.5	190	0.087	8.8×10^9

[a] The number of amide protons in class i.

nmr (and calculated) rate constants for the ring peptide may be the different experimental conditions employed for each method. The THX was performed near pH_{min} and the nmr experiments were performed away from pH_{min}, toward alkaline pH. If general acid and base catalysis or some other exchange process were significant near pH_{min}, then the rate constants measured by THX would be too large. The discrepancy between the THX and the nmr data might also be due to the existence of a different molecular form in the THX experiments since the former were done at a lower temperature and acid pH.

Despite the poor agreement between calculated and measured k_H and k_{OH} for the acyclic polymyxin B_1 ring peptide, the measured rate constants for polymyxin B_1 are in general agreement with the calculated constants and with the observed rate constants for poly-D,L-alanine and poly-D,L-lysine (originally thought to be random coils in solution) given in Table II. Therefore, polymyxin B_1 probably has neither an appreciable hydrogen bonded secondary structure nor sterically shielded amide protons in solution. This conclusion is consistent with dialysis data which show that although polymyxin B_1 dialyses as a compact peptide, it is not as compact as bacitracin A (Craig, 1964), which probably does have internally hydrogen bonded secondary structure (Galardy *et al.*, 1971).

TABLE III: Calculated Rate Constants for Specific Acid and Specific Base Catalyzed Exchange of the Amide Protons of Polymyxin B_1 and the Polymyxin B_1 Ring Peptide at 0°.[a]

Amide Proton	Log k_H^R − log k_H^{Ala}	Log k_{OH}^R − log k_{OH}^{Ala}	k_H (M^{-1} min^{-1})	k_{OH} (M^{-1} min^{-1})
Polymyxin B_1				
Dab^1	+0.71	−0.63	19	8.3×10^8
Thr^2	−0.80	+0.80	0.56	2.3×10^{10}
Dab^3	−1.00	+0.40	0.36	8.9×10^9
$Dab^4{}_\alpha$	−0.40	+0.30	0.14	7.1×10^9
$Dab^4{}_\gamma$	+1.14	−0.72	41	6.7×10^8
Dab^5	−0.55	+0.70	1.0	4.5×10^9
Phe^6	−0.60	+0.20	0.89	5.6×10^9
Leu^7	−0.30	+0.10	1.8	4.5×10^9
Dab^8	−0.55	+0.10	1.0	4.5×10^9
Dab^9	−0.95	+0.40	0.40	8.9×10^9
Thr^{10}	−0.70	+0.80	0.42	2.3×10^{10}
Polymyxin B_1 ring peptide				
$Dab^4{}_\gamma$	+0.59	−0.62	14	8.6×10^8
Dab^5	−2.20	+1.75	0.022	2.0×10^{11}
Phe^6	−0.60	+0.20	0.89	5.6×10^9
Leu^7	−0.30	+0.10	1.7	4.5×10^9
Dab^8	−0.50	−1.25	1.1	2.0×10^8
Thr^{10}	−2.45	+2.45	0.013	1.0×10^{12}

[a] The amino acid residues are numbered as in Figure 1. Log k^R − log k^{Ala} is defined as in Molday *et al.* (1972).

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