The Solution Conformation of the Ferrichromes

IV. pH DEPENDENCE OF THE INDIVIDUAL SLOW AMIDE HYDROGEN-DEUTERIUM EXCHANGE IN ALUMICHROME*

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SUMMARY

The kinetics of hydrogen-deuterium exchange for the four individual protected amides of alumichrome, the Al³⁺ analogue of ferrichrome, have been studied by proton magnetic resonance. In the range 3 < pD < 7 the exchange rates are relatively invariant. A tighter binding to the metal as the pD is raised results in a conformational stability gain, which compensates for the known base catalysis of the intrinsic amide hydrogen exchange within this pD range. The analysis of the exchange kinetic data versus temperature within the framework of the "absolute reaction rate theory" yields the enthalpy (ΔH°) and entropy (ΔS°) contributions to the free energy of activation (ΔF°). Depending on the particular amide, ΔH° and ΔS° appear to vary over wider ranges than does ΔF°. On the average it is found that raising the pD from ~3 to ~7 increases ΔH° while decreasing ΔS°. It is proposed that while conformational fluctuations are of importance at low pD, at neutrality the exchange of certain amides might proceed through a higher energy barrier without significant exposure to the solvent.

Proton magnetic resonance has recently proven to be an excellent tool for conformational analysis of small cyclic peptides, depsipeptides, and macrotetrolides (1-4). The increasing availability of stronger static magnetic fields has enabled resolution of many of the proton resonances of interest in these low molecular weight compounds. In particular, the identification of single amide NH resonances makes this spectroscopy especially useful to monitor the hydrogen-deuterium exchange at specific sites within the polypeptide.

Ferrichrome is a cyclohexapeptide of composition

\[ (\text{Gly}^1\text{-Gly}^2\text{-Gly}^3\text{-Orn}^3\text{-Orn}^2\text{-Orn}^1) \rightarrow \text{Fe}^{3+} \]

where Orn¹,²,³ represents δ-N-acetyl-δ-N-hydroxy-L-ornithyl (5, 6) and the residues are labeled as previously described (7). The metal is coordinated by the three hydroxamic acid ligands provided by the acylated δ-N-hydroxy ornithyl side chains. Ferrichrome acts as a growth factor for a number of microbes (6) and is presumably an iron carrier for Ustilago sphaerogena (8).

The conformation which has been proposed is depicted in Fig. 1. Ferrichrome thus possesses a compact, globular structure. The 3 consecutive substituted ornithyl residues have their side chains folded in a manner which optimizes octahedral coordination of the metal ion. The peptide backbone itself resembles an anti-parallel β-pleated sheet structure with the Orn³ and Gly³ residues paired by two carbonylamide transannular hydrogen bonds, as in the Schwzyer model for cyclohexapeptides (12). As can be deduced from the x-ray and PMR data, these are weak hydrogen bonds with the Orn³-NH...O=C-Gly³ bond (2.99 Å according to the x-ray) being more stable than the conjugated Orn³-C=O...HN-Gly³ bond. The amide hydrogens of glycyls 1 and 2 are exposed and free to interact (H-bond) with the solvent, while those belonging to the remaining ornithyl residues are either involved in a short (2.80 Å according to the x-ray), stable hydrogen bond directed to its own side chain N—O hydroxamate oxygen atom (Orn³), or buried in a pouch limited by the peptide backbone ring itself and the side chains of the ornithyl residues embracing the metal (Orn¹). The model depicted in Fig. 1 thus shows amide hydrogen atoms with different degrees of intramolecular hydrogen bonding (Orn³ > Orn² > Gly³) and steric shielding which ranges from complete exposure (glycyls 1 and 2) to significant occlusion in a hydrophobic environment (Orn¹).

Since ferrichrome is extremely soluble in water, no instrumental sensitivity problem handicaps its PMR study in aqueous solution. Furthermore, the high stability of the metal complex

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results in a very rigid structure which practically "freezes" the environment around each single proton, yielding excellent spectroscopic resolution of the six amide NH's whose resonances are spread over a range of ~4 ppm. Fortunately, the water (or HD0) resonance is sufficiently shifted to higher fields that it does not interfere with the detection of any of the amide absorption peaks. Fig. 2 shows the amide NH resonance region for alumichrome in aqueous solution, and the already demonstrated assignment of the absorptions to the corresponding residues (11).

The conformational state of the peptide can be drastically affected by the binding of the metal (7, 13). Emery (13) found that the bulk hydrogen-tritium exchange of the chelates (ferrichrome and ferrichrome A) was much slower than that of the deferri-peptides. Even though his data supported the x-ray model for crystalline ferrichrome A, direct assignment of the exchanging hydrogens to the residues in the peptide sequence is not possible from the data obtained by the Englander two-column gel filtration technique.

In this paper our interest is limited to the use of the slow amide hydrogen exchange kinetics in alumichrome as conformational probes and models for retarded amide hydrogen exchange in proteins. Hence no attention will be paid to the two relatively fast amides, namely, those of glycyl 1 and 2, since the knowledge that they are fast is sufficient information for these purposes. The temperature dependence of the exchange rate constants will be analyzed in the context of Eyring’s "Absolute Reaction Rate Theory" so that ∆S^‡ (the activation entropy) and ∆H^‡ (the activation enthalpy) will be estimated at different pD levels. From the data for each alumichrome amide the relative steric and hydrogen-bonding contributions to their hydrogen exchange retardation, and hence to the conformation of the molecule, will be discussed. Finally, the mechanistic implications for the hydrogen exchange of the amides will be analyzed within the contexts of Klotz’s "direct exchange" (14) and the Linderström-Lang (15) hypotheses at various pD levels.

**METHODS**

The alumichrome sample was prepared as previously described (7). The pD levels were calculated by adding 0.4 to the pH reading from a glass electrode pH meter (16). The solutions at pD 7.22 and 5.14 were buffered in 0.005 M sodium phosphate and 0.005 M sodium acetate, respectively; at lower pD levels no buffer was judged necessary because the peptide itself, through its chelated hydroxamate side chains, provided enough buffering capacity at the concentrations used. All pD levels were adjusted with concentrated NaOD and DCI.

In all experiments the spectrometer probe was pre-equilibrated to the desired temperature for at least 1 hour. Then the field homogeneity was adjusted with a sample of composition identical with the one to be studied. The sharp lines of the free methyls in the hydroxamate acyl groups provided an excellent internal standard for rapid tuning. Probe temperatures were determined with ethylene glycol. A Varian HR220 NMR spectrometer, which operates at 220 MHz, was used.

The hydrogen exchange experiments were initiated by dissolving 70 mg of peptide, preweighed in the NMR tube, in 0.5 ml of buffered 1H0, or DCI solution in 1H0, to give an approximately 0.125 M solution at the desired pD. When the conditions resulted in fast exchange, the sample-containing NMR tube was chilled during dissolution of the peptide and until its insertion in the spectrometer. The field homogeneity was then quickly readjusted with the fine controls; during the few minutes required by this process the sample would equilibrate to the probe temperature.

The exchange was followed in time by successive scanings of the amide proton resonance, the interval between scans depending on the exchange rate of the particular amide. Typically, an amide would be monitored for at least one exchange half-
time. The spectrometer gain was constant within the accuracy of our measurements.

The rate of exchange can be calculated either from the time dependence of the integrated amide proton resonance area or its peak amplitude. The second method was chosen because of drifts in the spectrometer integrator. When monitoring the exchange kinetics of a particular set of amides under constant pH and temperature, the radiofrequency power, receiver gain, amplification, and noise filtering were kept constant so that the uncertainty introduced by electronic noise was practically the same for all points in a kinetic curve.

RESULTS

The semilogarithmic plots of amide NH peak amplitude versus time were linearly least squares fitted by giving the same weight to each point. The slopes of the line yield \( k \), the first order exchange rate constant. The rate constants were then plotted semilogarithmically versus inverse temperature. The Eyring plots were least squares fitted, the weight for each point being given by the inverse logarithmic standard error of the corresponding \( k \).

Fig. 3 shows the kinetic plot for the slow amides of alumichrome at pH 5.14 and 24.2°C. This and similar plots yield the first order rate constants for hydrogen exchange for each of the slow amides studied under their particular pH and temperature conditions. The values so determined, with their standard deviations, are given in Table I.

Fig. 4 depicts Eyring plots for the rate constants given in Table I. The \( \Delta H^\dagger \) (slope) and \( \Delta S^\dagger \) (intercept) values, together with their standard deviations, are given in Table II. These figures summarize all of the kinetic data provided by the first order exchange curves of the type exemplified in Fig. 3 which are not shown individually.

Except for the case of alumichrome at pH 3.23, where the relatively higher ionic strength of the solution resulted in a poorer balancing of the spectrometer probe, the standard errors of the slopes of the kinetic curves \( (k) \) are relatively small. However, the dispersions of the Eyring plots are larger. Since the experimentally accessible temperatures are within the range \( 3.66 \times 10^{-2} > T^{-1} > 2.68 \times 10^{-4} \text{K}^{-1} \), it is to be expected that the standard deviations in the slope \( (\Delta H^\dagger) \) would be smaller than in the intercept \( (\Delta S^\dagger) \), calculated at the rather removed point \( T^{-1} = 0 \text{K}^{-1} \). Furthermore, the relative errors for \( \Delta S^\dagger \) are larger than for \( \Delta H^\dagger \) because the absolute values of \( \Delta S^\dagger \) are closer to zero.

Obviously, in the discussion that follows any conclusion based on the comparative values of the kinetic parameters will be valid within the accuracy of their determinations. In most cases the experimental uncertainties are small enough for our purposes; cases where this is not so will be noted explicitly.

DISCUSSION

General Kinetic Analysis—Hvidt and Nielsen have extended an early proposal of Linderström-Lang (17) and of Berger and Linderström-Lang (18) to rationalize protein hydrogen exchange in general (15). The molecule is assumed to fluctuate between more or less folded conformations (N states) in which the labile hydrogens are unexchanged and buried, and more or less relaxed conformations (I states) in which the labile hydrogen is unexchanged but exposed to the bulk solvent. It is assumed that the concentrations of the various protein conformations are stationary in the exchanging solution and that chemical equilibrium holds.

\[ \text{N} \xrightleftharpoons[k_1]{k_2} \text{I} \]

In the I conformations hydrogen exchange can readily take place with a first order rate constant, \( k_n \), whose magnitude approximates the first order exchange rate constants for low molecular weight compounds under similar conditions. Furthermore, it is assumed that the conformational drifts between the N and I states are characterized by first order rate constants, \( k_n \) and \( k_i \).

Along these lines the unfolding process in the alumichromes can be thought of as a substitution of the trivalent metal, ionically coordinated to the three hydroxamate ligands, by one, two, or three protons.

Unfortunately, pK values for these equilibria have not been measured. As judged from the acetyl hydroxamate stability constants, alumichrome should be a weaker complex than fer-
The first order rate constants ($k$) for the hydrogen-deuterium exchange of the slowly exchanging amides of alumichrome. The values are calculated for the individual amides from the slopes of the linear least squares fits of the experimental data points $t$ in Fig. 3. The $k$ values together with their standard deviations are tabulated versus temperature ($T$) at each pD. The pD levels are $5.04$ (b), 7.14 (c), and 7.22 (d). The units for $k$ are minutes$^{-1}$ for $k$ and °C for $T$. Each particular $k$ reflects a linear fit of a number of points which typically varied from 5 to 10 depending on the particular amide, temperature, and pD. Because the exchange of Gly$^3$ at pD 7.22 is too fast, it was not monitored and the corresponding data are absent in d.

| T | Gly$^3$ | Orn$^1$ | Orn$^2$ | Orn$^3$ |
|---|---------|---------|---------|---------|
| (a) pD 3.23 | | | | |
| 4.7 | 0.0041±0.0002 | 0.0164±0.0007 | 0.0202±0.0011 | 0.0190±0.0011 |
| 9.6 | 0.0207±0.0009 | 0.0184±0.0027 | 0.0332±0.0004 | 0.0210±0.0018 |
| 12.8 | 0.0300±0.0023 | 0.0243±0.0011 | 0.0305±0.0006 | 0.0291±0.0008 |
| 17.3 | 0.0396±0.0011 | 0.0536±0.0026 | 0.0797±0.0025 | 0.0723±0.0022 |
| 24.2 | 0.0761±0.0016 | 0.0660±0.0047 | 0.1276±0.0073 | 0.1796±0.0046 |
| 29.5 | 0.1377±0.0038 | 0.1470±0.0077 | 0.2186±0.0172 | 0.1917±0.0161 |
| 34.0 | 0.2455±0.0135 | 0.1470±0.0077 | 0.2186±0.0172 | 0.1917±0.0161 |
| (b) pD 5.04 | | | | |
| 17.3 | 0.0282±0.0004 | 0.0010±0.0001 | 0.0019±0.0001 | 0.0012±0.0001 |
| 24.2 | 0.0300±0.0011 | 0.0033±0.0001 | 0.0053±0.0001 | 0.0036±0.0000 |
| 30.5 | 0.0075±0.0017 | 0.0012±0.0003 | 0.0126±0.0003 | 0.0091±0.0002 |
| 34.0 | 0.1152±0.0015 | 0.0076±0.0006 | 0.0169±0.0007 | 0.0107±0.0004 |
| 41.2 | 0.2073±0.0062 | 0.0100±0.0005 | 0.0377±0.0016 | 0.0230±0.0007 |
| 51.0 | 0.0425±0.0169 | 0.0536±0.0010 | 0.1113±0.0053 | 0.0744±0.0064 |
| (c) pD 7.14 | | | | |
| 17.3 | 0.0557±0.0013 | 0.0000±0.0001 | 0.0010±0.0001 | 0.0010±0.0001 |
| 24.2 | 0.0984±0.0020 | 0.0020±0.0001 | 0.0033±0.0000 | 0.0033±0.0000 |
| 30.5 | 0.1852±0.0048 | 0.0033±0.0001 | 0.0062±0.0001 | 0.0062±0.0001 |
| 34.0 | 0.3668±0.0119 | 0.0042±0.0001 | 0.0082±0.0001 | 0.0107±0.0009 |
| 41.2 | 0.4792±0.0315 | 0.0104±0.0009 | 0.0217±0.0014 | 0.0204±0.0011 |
| 51.0 | 0.0316±0.0013 | 0.0086±0.0012 | 0.0632±0.0029 |
| (d) pD 7.22 | | | | |
| 5.0 | 0.0065±0.0001 | 0.0197±0.0004 |
| 9.6 | 0.0085±0.0002 | 0.0277±0.0005 |
| 12.8 | 0.0174±0.0004 | 0.0572±0.0151 |
| 17.3 | 0.0006±0.0000 | 0.0345±0.0009 | 0.1229±0.0045 |
| 24.2 | 0.0017±0.0000 | 0.0476±0.0017 | 0.2325±0.0063 |
| 30.5 | 0.0040±0.0001 | 0.1085±0.0035 | 0.3921±0.0179 |
| 41.0 | 0.0124±0.0002 | 0.2186±0.0064 | 0.5671±0.0320 |
| 41.2 | 0.0179±0.0005 | 0.4495±0.0226 |
| 51.0 | 0.0545±0.0033 |

For ferrichrome, Anderegg et al. (20) have determined $pK_1 = 1.49$. Hence, around pH = 3, about 0.5% hydroxamate protonation on the ornithyl side chains should be expected in ferrichrome, and even more in alumichrome. Our observation of a rapid exchange of Al$^{3+}$ for Fe$^{3+}$ at pH 3 demonstrates its existence (7). Furthermore, the PMR spectrum of alumichrome at this low pD shows the presence of extra peaks in the amide region, which exchange with deuterium at relatively faster rates and which can be assigned to the metal-free peptide.

The bulk hydrogen exchange kinetics of ferrichrome and alumichrome show that on lowering the pH (pD) from about 7 to about 3 the number of slowly exchanging hydrogens increases for both compounds (Table III). The data show that at neutrality alumichrome exchanges slower than ferrichrome and that the kinetic difference tends to disappear (a reversal is hinted) as the pH is lowered. This behavior suggests that the rate constants for the ligand-metal$^{3+}$ complex dissociation might be smaller for Al$^{3+}$ than for Fe$^{3+}$ near pH (pD) 7 and that the stability of the Al$^{3+}$ complex is more dependent upon $H^+$ ($D^+$) concentration than that of the ferric analogue. This is supported by our observation that at neutrality no exchange of Al$^{3+}$ for Fe$^{3+}$ is detected, while it is known that $^{57}$Fe$^{3+}$ readily exchanges with ferrichrome (about 8 min for half completion) at pH 6.3 and 37° (21). The differences in the hydrogen exchange kinetics should not be attributable to isotope effects since Emery verified that rates for hydrogen-tritium exchange do not differ significantly from those for deuterium-tritium exchange. Furthermore, throughout our experiments we observed no changes in the positions of the NH resonances as deuteration was proceeding, indicating that the conformation of the molecule is insensitive to the hydrogen isotope at the amide.

Since hydrogen exchange in the presence of excess Fe$^{3+}$ did not affect the kinetics of the process, Emery suggested that local environmental factors rather than a conformational “breathing” process would dominate the observed exchange rates. However, a metal-free intermediate could be of negligible importance as a contribution to the conformational fluctuations responsible for the observed exchange. Thus, random modulation of the distance between the metallic center and each side chain bidentate might result in short-lived di- and eventually monohydroxamate complexes which could account for the “unfolded,” loosely structured intermediates responsible for most of the measured exchange. If such were the case, the kinetics of the folding process would be practically independent of the excess metal ion concentration. Furthermore, the effect of excess metal would be obscured by the formation of 1:1 complexes, thus opening the molecule (22).

Following the nomenclature of Hvilsted and Nielsen (15), the equilibrium between the "native" (the hexadentate chelate) peptide and any and all of its “unfolded” (partially or totally monochelated) forms can be represented as N and I states, respectively. We propose that an increased proton concentration shifts the equilibrium to the right both by increasing $k_1$ and by decreasing $k_2$. It should be obvious, however, that in general the $k_1$: $k_2$ ratios as well as the conformational change contributions to the free energy of activation of the exchange reaction will be different for each of the amides in the molecule.

The rate of exchange for a free amide hydrogen is known to be at a minimum at about pH 3 (15, 23). However, upon going from pD 5.14 to 3.23 the rate of hydrogen exchange for all of the alumichrome ornithyl amides increase from exchange half-times of 416, 220, and 219 min to 12.3, 8.0, and 9.3 min for Orn$^1$, Orn$^2$, and Orn$^3$, respectively (Table II). In contrast, the exchange...
Alumichrome

Fig. 4. Eyring plots for the alumichrome Gly3, Orn1, Orn2, and Orn3 amide hydrogen-deuterium exchange kinetics at pD 3.23 (a), 5.04 (b), 5.14 (c), and 7.22 (d). At pD 7.22, Gly3 exchanges too fast to be monitored by this technique. The lines are weighted linear least squares fits of $-R \ln \left( \frac{h}{kT}k \right)$ in cal/(K x mole) versus inverse temperature in (°K⁻¹). The weight for each point is given by the inverse logarithmic standard deviation of the corresponding $k$ determined from plots such as shown in Fig. 3. The slopes of these linear plots yield $\Delta H^\ddagger$, the enthalpy of activation, while the intercept (at $T^{-1} = 0$) yields $\Delta S^\ddagger$, the entropy of activation. $\Delta H^\ddagger$ and $\Delta S^\ddagger$ so determined, together with their standard deviations, are given in Table II. At pD 3.23 the linear fits are poor, mainly because of lower instrumental signal-to-noise due to the higher ionic strengths of the solutions as discussed in the text. However, the data in a clearly demonstrate a closer kinetic equivalence between the different amides than at lower acidities (b, c, and d). The labeling of the points, according to the key included in a, follows the same convention as in the previous figure.

half-time for Gly3 does not change significantly, from 6.2 to 7.5 min. A simple explanation can be given for these effects; on lowering the pD the intrinsic rate of amide hydrogen exchange drops, but the stability of the chelate is so reduced as to more than compensate for this effect and the over-all kinetics is accelerated. For Gly3 an almost exact compensation results so that no major kinetic change is observed. At about pD 3, stability differences due e.g. to intramolecular hydrogen-bonding are of little influence since the exchange proceeds mainly through the relatively abundant unfolded conformation. At pD 5.14, the over-all ground state conformation is enforced and small stabilizing differences such as intramolecular hydrogen-bonding would show more pronounced relative effects. On raising the pD from 5.14 to 7.22 the stability of the chelate is increased further and these effects become even more characteristic for each amide.

At pD 7.22 intrinsic rates of amide hydrogen exchange due to base catalysis are relatively large. A change in pD from 5.14 to 7.22 results in a 20- and 75-fold increase in hydrogen-deuterium exchange exchange rates for Orn2 and Orn3, respectively, while Gly3, exhibiting a half-life of 6.2 min at the lower pD, exchanges immeasurably rapidly at neutrality. This pD change, however, does not appear to affect significantly the exchange rate of Orn1, whose exchange half-time changes from 416 min (pD 5.14) to 362 min (pD 7.22). Thus, while at pD 3.23 the four slowly exchanging amides exhibit rate constants of the same order of magnitude, at pH 5.14 the observed $k$ for Gly3 is about 10^4 larger than for the ornithyl amides and, at neutrality, the orders of the observed rate constants differ one from the other by at least one order of magnitude ($2.4 \times 10^{-7}$, $7.0 \times 10^{-3}$, and $1.9 \times 10^{-8}$ min⁻¹ for Orn3, Orn2, and Orn1, respectively).

The amplification of the kinetic differences among the four
The activation enthalpy $\Delta H^\ddagger$ and the activation entropy $\Delta S^\ddagger$ for the amide hydrogen-deuterium exchange of aluminichrome at pD 3.23 (a), 5.04 (b), 5.14 (c), and 7.22 (d). The kinetic data prove useful in pointing out conformational differences between Gly$^3$ and Orn$^3$. Since $\Delta H^\ddagger$ for Gly$^3$ is about 5.0 Cal below the value for Orn$^3$ (= 20.9 Cal) and hydrogen bond energies are of the order of 3 to 8 Cal per mole (25), the enthalpy difference between these two amides may well account for the contributions that hydrogen bond stabilization would make. Leichtling and Klotz (26) have discussed the importance of the temperature dependence of the dissociation of $\text{D}_2\text{O}$ and of the hydroxamate binding constant have, perforce, been ignored. Correcting for the enthalpy of ionization of water would shift the same amount all of the tabulated $\Delta H^\ddagger$ values for the amides while leaving unaffected their differential value. Furthermore, as discussed above, the dissociation of the hydroxamate complex is the main contribution to the peptide unfolding process and hence its temperature dependence does not need to be accounted for separately. Thus, although the data represent the gross exchange process, they still enable useful conclusions to be made regarding the overall trends of the kinetics and, in particular, the extent to which conformational factors participate in the exchange mechanism.

On going from pD 5.14 to 3.23, $\Delta H^\ddagger$ and $\Delta S^\ddagger$ decrease about 6 Cal and 13 e.u., respectively, for the ornithines, while for Gly$^3$ $\Delta H^\ddagger$ increases 4 Cal and $\Delta S^\ddagger$ 13 e.u. This suggests a different nature for the exchange mechanism of these two types of amides. Raising the pD from 5.14 to 7.22 appears not to affect $\Delta S^\ddagger$ appreciably either for Orn$^3$ or for Orn$^3$, the increased rate of exchange for these resulting from a 1 to 2 Cal decrease in $\Delta H^\ddagger$. However, the same increment in basicity increases $\Delta H^\ddagger$ by about 4 Cal and $\Delta S^\ddagger$ by about 13.5 e.u. for Orn$^3$.

At pD 5.14, Table II shows that the $\Delta S^\ddagger$ values are all negative, decreasing in the order Orn$^3$ ($-4.7$ e.u.) $>$ Orn$^3$ ($-5.8$ e.u.) $>$ Orn$^3$ ($-9.3$ e.u.) $>$ Gly$^3$ ($-17.1$ e.u.), which might indicate a trend of decreasing accessibility of the particular hydrogen. The $\Delta H^\ddagger$ values show a parallel increase so that at 25$^\circ$ the three ornithyl amides have about the same free energies of activation ($\Delta F^\ddagger = 23.3$ to 23.7 Cal) while the Gly$^3$ is about 2 Cal ($\Delta F^\ddagger = 21.2$ Cal) below these values.

The kinetic data prove useful in pointing out conformational differences between Gly$^3$ and Orn$^3$. Since $\Delta H^\ddagger$ for Gly$^3$ is about 5.0 Cal below the value for Orn$^3$ (= 20.9 Cal) and hydrogen bond energies are of the order of 3 to 8 Cal per mole (25), the enthalpy difference between these two amides may well account for the contributions that hydrogen bond stabilization would make. Leichtling and Klotz (26) have discussed the importance of inductive effects on the amide exchange stability. At 25$^\circ$ the pK$\alpha$ values of ornithine are 1.94 and 8.65 and of glycine 2.34 and 9.60 (27). This indicates that any differential inductive effects would tend to increase the positive charge on the ornithyl relative to the glycyl amide NH and C=O and hence would result in a relative increase in the electrostatic contribution to the strength of the Orn$^3$ cross-amide hydrogen bond while increasing its intrinsic rate of base-catalyzed hydrogen exchange.

### Table II

| Component | Value (Cal per mole) | Component | Value (Cal per mole) | Component | Value (Cal per mole) |
|-----------|---------------------|-----------|---------------------|-----------|---------------------|
| Acid      | 24                  | Base      | 21                  |
| Orn$^3$   | 12                  | Orn$^3$   | 5                   |
| Gly$^3$   | 20                  | Gly$^3$   | 20                  |

These values will be modified when considering the exchange behavior of amides within a peptide or protein in its native state; H bond and steric shielding effects will contribute both to $\Delta H^\ddagger$ and $\Delta S^\ddagger$ since the transition state I might require H bond breakage and even partial unfolding of the structure. Furthermore, in the discussion that follows, both the temperature dependences of the dissociation of $\text{D}_2\text{O}$ and of the hydroxamate binding constant have, perforce, been ignored. Correcting for the enthalpy of ionization of water would shift the same amount all of the tabulated $\Delta H^\ddagger$ values for the amides while leaving unaffected their differential value. Furthermore, as discussed above, the dissociation of the hydroxamate complex is the main contribution to the peptide unfolding process and hence its temperature dependence does not need to be accounted for separately. Thus, although the data represent the gross exchange process, they still enable useful conclusions to be made regarding the overall trends of the kinetics and, in particular, the extent to which conformational factors participate in the exchange mechanism.
This reinforces the suspicion that while Orn3 is transannularly hydrogen-bonded, Gly3 might not be in this state. Why then the relative protection of the Gly3 NH? The answer is given from the ΔS↑ differences. The Gly3 NH is about 11.3 e.u. (= 3.4 Cal at 25°) more stable than the Orn3 NH, suggesting that indeed steric effects are relatively more important for the glycyl than for its paired ornithyl.

At pD 3.23 and 25° the ΔF↑ values are similar for all of the slow amides, ornithyls, and glycine alike. The large negative ΔS↑ value found for the ornithyl residues probably indicate that their exchange is proceeding through a different conformation, favored by relatively lower ΔH+ values (16.3 Cal vs. 115.6 Cal). At this lower pD the ornithyl amides provide a clearer picture of the molecular fluctuations than do the figures for the Gly4 exchange, not only because of a relatively better fit of the experimental points, but also because they are more directly sensitive to the pH-dependent metal chelation.

Mechanistic Implications—By calculating the exchange rate constant k1 for a random polypeptide, namely, poly(DL-alanine), on the basis of Equation 2 and dividing the observed k by this number, a gross estimate can be obtained of the relative impediment to exchange for the different amides under the various pH conditions. It is within the framework of the Lindermuth-Lang, Hvidt, and Nielsen treatment that the meaning of k1/k2 becomes mechanistically meaningful (15). According to the theory, if the exchangeable amide hydrogens do not remain too long in the exposed state (k1 < k2), two extreme limits are of interest, the unimolecular EX1 (k1 < k2 << k3) and the bimolecular EX2 (k1 << k2 << k3) mechanisms. In the EX1 mechanism the rate-limiting step is the unfolding of the native structure and the observed k equals k1, while in the EX2 mechanism the peak-out k process is weighted by the extent of unfolding of the peptide and the observed k equals (k1/k2)k2.

For alumichrome at pD 3.23 and 25°, 0.3 ≳ k/k2 ≳ 0.5 for the slow amides, which is too large for an EX1 mechanism. By contrast, the EX2 mechanism would imply k = k1 << k2 which is contradictory since k2 ≳ 0.2 min⁻¹ (poly(DL-alanine)) and the observed k ≳ 0.4 min⁻¹. It is then likely that at this pH the actual mechanism be intermediate between the extremes EX1 and EX2, namely, k1 < k2 ≳ k2.

From the experimental pH dependence of the hydrogen exchange rate constant above pH 4 for poly(DL-alanine) (Equation 2) it can be derived that:

$$\left(\frac{\partial \log k}{\partial \text{pD}}\right)_T = 1$$

By assuming k1 and k2 to be relatively pH independent, it can be shown that an EX mechanism should yield:

$$\left(\frac{\partial \log k}{\partial \text{pD}}\right)_T = 1$$

This analysis was applied to alumichrome at pD 5.14 by studying the exchange kinetics at pH 5.04. The data at these two pD levels as shown in Table I and Fig. 4, may be compared. A Δ(pD) = -0.1 results in about a doubling of the hydrogen exchange rate of the ornithyl amides, while the Gly4 rate is halved, i.e.:

$$\left(\frac{\partial \log k}{\partial \text{pD}}\right)_T = -1.6 \text{ for Gly4}$$

Thus, while Gly3 seems to satisfy the above criterion and might exchange through an EX1 mechanism, the data are less clear for the ornithyl amide mechanism. This minor pH shift appears to affect the Gly4 and Orn values of ΔH↑ and ΔS↑ in different ways. In the case of Gly4 the decrease in the exchange rate is due to a 6.5 e.u. drop in ΔH↑ that overcomes the opposing 1.5 Cal drop in ΔS↑. The ornithyl amides, however, decrease their exchange rate due to slightly more favorable values of both ΔH↑ and ΔS↑. These changes for the ornithyl amides, although small, are in the direction that would be expected from an increase in the acid-catalyzed metal exchange as discussed when considering the exchange kinetic data for pH 3.23. By contrast, changes in the Gly4 amide kinetic parameters, in accordance with the k2 dependence on pD, are suggestive of a tighter coupling of this amide to a relatively more pH invariant peptide backbone conformation. The k1/k2 ratios, however, although larger for Gly3 than for the ornithyl amides, are all of the right order of magnitude for an EX1 exchange mechanism at this pD. In summary, it is proposed that the reason why the above differential criterion does not apply for the ornithyl amides is that their k1 and k2 values are so dependent on the acidity of the medium that they fall outside its range of applicability.

On raising the pH of 5.14 to 7.22, metal chelation-dependent k1 decreases and k2 increases. The ratios k1/k2 now yield the values 0.36 × 10⁻⁵, 9.54 × 10⁻⁵, and 32.9 × 10⁻⁵ for Orn1, Orn2, and Orn3, respectively. The range covered is about two orders of magnitude, suggesting now more differentiated exchange pathways within the applicability of an EX2 mechanism, if it applies to them all. Indeed, at pH 7.22, k2 = 7.4 × 10⁶ and it is likely that an EX mechanism (k1 < k2 << k3) is responsible for the exchange. For Orn1 at pH 7.22, however, we find a ΔS↑
of about 4 e.u. per mole. There would thus be little or no change in the "ordering" of the system in going through the activated state. Hence, it is suggested that at neutral pH the exchange proceeds through direct interaction between the exchanging species, i.e. without involving any major unfolding to expose the buried amide. The high ΔH* (~25 Cal per mole) is easily understood as the barrier for the aqueous hydroxyl to reach the buried Orn NH site should be high. According to the mechanism of Berger et al. (28), the base-catalyzed hydrogen exchange involves the existence of an anionic intermediate. Hence, the Orn amide, by finding itself buried in a highly nonpolar surrounding, would show a further reduction of its rate of hydrogen exchange due to a relative destabilization of the charged intermediate (14, 26, 29).

Gramicidin S-A versus Alumichrome—The bulk 1H-1H exchange of gramicidin S-A has been studied by rapid dialysis and correlated with PMR data in low polarity solvents by Craig and collaborators (30, 31). Linear interpolation of the gramicidin S-A 0° rate constants to pH 3.23 yields a half-life of hydrogen exchange of about 2500 min. If one calculates these same kinetic parameters for the slow amide hydrogen of alumichrome at 0° from the ΔH* and ΔS* values at pH 3.23 (Table III), the figures for the exchange half-lives are:

| Gly | Orn | Orn | Orn |
|-----|-----|-----|-----|
| 180 | 126 | 98  | 126 |

For poly(Al-alanine) half-lives of ~68 min are calculated for these same conditions. It is then clear that although some kinetic stabilization of alumichrome relative to poly(Al-alanine) might be present, under these conditions the cyclic decapptide would appear to have a more rigid conformation, the difference in ΔS* being about 1.2 Cal. An explanation for this must be sought in the different mechanisms through which these two peptides exchange, as in contrast with alumichrome the conformation of gramicidin should be essentially insensitive to pH.

In alumichrome the reduced rate of amide hydrogen exchange, even at pH 3.14 and 0°, could still be accounted for in terms of some conformational rigidity conferred to the peptide by the metal. Hence, we considered it convenient to observe the exchange kinetics of the metal-free peptide. The exchange of the three resolved PMR bands in deferriferichrome (7) was followed at pH 3.0 and 3.5°. The measured half-lives are:

| Band 1 (Gly) | Band 2 (Gly+Gly+Orn) | Band 3 (Orn+Orn) | Poly(Al-alanine) |
|--------------|-----------------------|------------------|------------------|
| 62 ± 2       | 80 ± 2                | 84 ± 2           | 19               |

The values for poly(Al-alanine) were calculated as before. Although the data suggest a more protected location for the amides with resonances at higher fields, the protection is not great. It is then quite likely that in aqueous solution the preferred conformation does not have internal amides in sufficient relative concentration to be PMR detectable. Such an idea has been advanced in regard to the lack of hydrogen exchange kinetic discrimination between PMR observable amide hydrogens in synthetic cyclic hexapeptides (32, 33). It should be noted, however, that at higher pH levels the exchange in gramicidin S-A is much faster than in alumichrome. The structural stabilization conferred by the metal chelate moiety clearly differentiates the two compounds.

Conclusions—A merit of alumichrome as a model for amide hydrogen exchange is that it shows the relative requirement of both the Klotz and the Linderström-Lang approaches for explaining the observed kinetics. Both local environmental effects and conformational fluctuations are present, the relative con-
In Fig. 5 the relative trends of the $\Delta F_1^+$ and the $\Delta F_2^+$ barriers with $pD$ are depicted. At $pD \sim 3$ the $\Delta F_1^+$ barrier is so low that the overall exchange rate is determined by the $\Delta F_1^+$ step, the intrinsic rate of amide hydrogens-deuterium exchange being minimal. At $pD \sim 7$, the structure becomes reinforced because of the high kinetic stability of the chelate moiety. Even though $\Delta F_2^+$ is now relatively low due to the base catalysis, the height of the $\Delta F_2^+$ barrier results in measurably low exchange rates. At $pD \sim 5$ the situation is intermediate between the two previous cases. Even though base catalysis should result in a relatively fast intrinsic exchange, the structure is more stabilized by the $\Delta F_2^+$ trihydroxamate complex than at lower $pD$, and some exchange retardation becomes apparent. Although at this $pD$ the EX$_3$ mechanism may be predominant, a $k_i$-controlled exchange may commence to contribute to the measured rates.

One would expect that any conformational fluctuations present at $pD \sim 5$ will also be present at lower $pD$, in addition to those controlled by the metal. The $\Delta S^+$ values for Orn$^1$, Orn$^2$ and Orn$^3$ at $pD 7.22$ show that these extra contributions to the conformational fluctuations are small. This suggests a lack of flexibility for the peptide backbone ring per se and that the whole molecule is tightly structured once the metal exchange rate is reduced.

Out of this investigation, a picture emerges that provides us with a molecular dynamics view of the ferrichrome solution conformation.

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