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THE SOLUTION CONFORMATION OF THE FERRICHROMES, IV: pH dependence of the individual slow amide hydrogen-deuterium exchange in alumichrome; kinetic analysis and conformational implications.*

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Summary: The kinetics of hydrogen-deuterium exchange for the four individual protected amides of alumichrome, the Al^{+3} 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 ($\Delta H^{\dagger}$) and entropy ($\Delta S^{\dagger}$) contributions to the free energy of activation ($\Delta F^{\dagger}$). Depending on the particular amide, $\Delta H^{\dagger}$ and $\Delta S^{\dagger}$ appear to vary over wider ranges than does $\Delta F^{\dagger}$. On the average it is found that raising the pD from $\sim$3 to $\sim$7 increases $\Delta H^{\dagger}$ while decreasing $\Delta S^{\dagger}$. 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.

Introduction

Proton magnetic resonance (PMR) 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.

A successful application of the amide hydrogen exchange PMR method, which exemplifies its value in a conformational study of a simple peptide, is the work on gramicidin S-A by Stern, Gibbons and Craig (5). These authors found that while in CD_3OD and in $(CD_3)_2SO$ + 5% D_2O the leucine and valine amides exchange with half times of one and at least two weeks, respectively, in CD_3OD ornithine and phenylalanine exchange relatively rapidly ($t_{1/2}$ = 0.5 hr). The agreement of these exchange times with the stabilization to be expected from intramolecular hydrogen-bonding on the basis of the Hodgkin-Oughton-Schwyzer model (6,7) is excellent and affords valuable evidence to support the antiparallel β-pleated structure proposed for this cyclodecapeptide. Unfortunately, these exchange rates cannot be extended directly to the interpretation of the hydrogen exchange of peptides and proteins in water. The PMR studies required solvents of relatively low polarity in order to have sufficient peptide concentration for a good signal sensitivity. The correspondence of these data with the bulk hydrogen-tritium exchange study of this peptide in aqueous solution, accomplished in the same

laboratory at low temperatures and at pH around 3 by rapid dialysis techniques (8), is still subject to experimental verification.

Ferrichrome is a cyclohexapeptide of composition $(\overleftarrow{\text{-Gly}^3\text{-Gly}^2\text{-Gly}^1\text{-Orn}^3\text{-Orn}^2\text{-Orn}^1}\text{-})^{-3} \cdot Fe^{+3}$, where $Orn^{1,2,3}$ represents δ-N-acetyl-δ-N-hydroxy-L-ornithyl (9,10) and the residues are labeled as previously described (11). 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 (10) and is presumably an iron carrier for Ustilago sphaerogena (12).

The conformation of ferrichrome and of related seryl-containing peptides has been well characterized both by X-ray (13,14) and PMR (11,15,16) studies. The substitution of Al^{+3} or Ga^{+3} for Fe^{+3} was necessary in the PMR work to eliminate line broadening by the paramagnetic ion. We have already discussed the conformational similarity between these complexes (11,16), while Emery has found that both the Al^{+3} ("alumichrome") and Ga^{+3} ("gallichrome") analogues of ferrichrome are biologically active (12).

Fig. 1

The conformational model which has been proposed is depicted in Fig. 1. Ferrichrome thus possesses a compact, globular structure. The three 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 Orn^3 and Gly^3 residues paired by two carbonyl-amide transannular hydrogen bonds, as in the Schwyzer model for cyclohexapeptides (7). As can be deduced from the X-ray and PMR data, these

are weak hydrogen bonds with the Orn^3-NH···O=C-Gly^3 bond (2.99 Å according to the X-ray) being more stable than the conjugated Orn^3-C=O···HN-Gly^3 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 sidechain N-O hydroxamate oxygen atom (Orn^2), or buried in a pouch limited by the peptide backbone ring itself and the side chains of the ornithyl residues embracing the metal (Orn^1). The model depicted in Fig. 1 thus shows amide hydrogen atoms with different degrees of intramolecular hydrogen bonding (Orn^2 > Orn^3 > Gly^3) and steric shielding which ranges from complete exposure (glycyls 1 and 2) to significant occlusion in a hydrophobic environment (Orn^1).

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 ($K \sim 10^{30}$) 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 HDO) 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 assignment of the absorptions to the corresponding residues along the peptide backbone. Ferrichrome appears thus as an ideal compound for PMR hydrogen exchange studies. Its conformational

Fig. 2

state can be drastically affected by the binding of the metal and its high solubility in water enables study of the exchange kinetics directly in this solvent.

Emery (17) found that the bulk hydrogen-tritium exchange of the chelates (ferrichrome and ferrichrome A) was much slower than that of the deferri-peptides, thus indicating the conformational stabilization conferred by metal-binding. 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, as in the case of the similar bulk exchange experiment on gramicidin S-A mentioned above.

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 glycyls 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 $\Delta S^{\dagger}$ (the activation entropy) and $\Delta H^{\dagger}$ (the activation enthalpy = the "heat of activation") will be estimated at different pD's. 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" and the Linderstrøm-Lang hypotheses at various pD's.

Methods

The alumichrome sample was prepared as previously described (11). The pD's were calculated by adding 0.4 to the pH reading from a glass electrode pH-meter (18). The solutions at pD 7.22 and 5.14 were buffered in 0.005 molar sodium phosphate and 0.005 molar sodium acetate, respectively; at lower pD's 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's were adjusted with concentrated NaOD and DCl.

In all experiments the spectrometer probe was preequilibrated to the desired temperature for at least one hour. Then the field homogeneity was adjusted with a sample of composition identical to 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, pre-weighed in the NMR tube, in 0.8 ml of buffered D_2O, or DCl solution in D_2O, to give a $\sim$ 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 scannings of the amide proton resonance, the interval between each scan depending on the exchange rate of the particular amide. Typically, an amide would be monitored for at least one exchange half-time. A main concern was the instability of the field homogeneity when the exchange required more than 10 to 15 minutes. Experience showed that retuning the field homogeneity within this interval was necessary in order to obtain good first order decay plots. 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 pD 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 *vs.* 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 exchange half-times were calculated on the basis that $k = 0.69/t_{1/2}$. The rate constants were then plotted semilogarithmically *vs.* inverse temperature as required by the "Absolute Reaction Rate Theory" expression:

$$R \cdot \ln\left(\frac{h}{KT}\, k\right) = \Delta S^{\dagger} - \frac{\Delta H^{\dagger}}{T}$$

where R (gas constant) = 1.987 $cal \cdot deg^{-1} \cdot mole^{-1}$

h (Plank's constant) = 1.104×10^{-28} $erg \cdot min$

K (Boltzman's constant) = 1.380×10^{-16} $erg \cdot deg^{-1}$

k (exchange rate constant) = measured in min^{-1}

and T (temperature) = measured in ^{o}Kelvin

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

Figure 3 shows the kinetic plot for the slow amides of alumichrome at pD 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 pD and temperature conditions. The values so determined are given in Table I. The standard deviations shown in this table reflect the accuracy of the linear fits.

Table I

Figure 4 depicts yring 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 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 pD 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, reflecting perhaps poorer definitions of the temperature

and/or pD values for the individual data points. Since the experimentally accessible temperatures are within the range $3.66 \times 10^{-3} > T^{-1} > 2.68 \times 10^{-3}$ °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 $T^{-1} = 0$ °K^{-1}. Furthermore, the relative standard deviations for $\Delta S^{\dagger}$ are larger than for $\Delta H^{\dagger}$ because the absolute values of $\Delta S^{\dagger}$ are closer to zero.

Fig. 4

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 as reflected by the corresponding standard deviations. In most cases the experimental uncertainties are small enough for our purposes; cases where this is not so will be noted explicitly.

Discussion

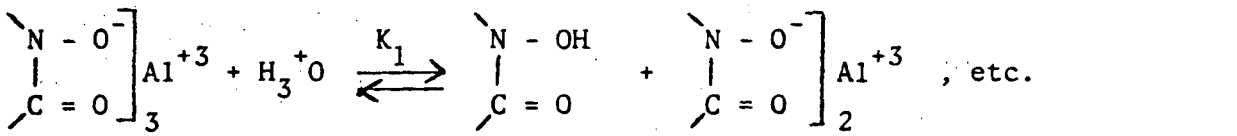
General kinetic analysis

Hvidt and Nielsen have extended an early proposal of Linderstrøm-Lang (19) and of Berger and Linderstrøm-Lang (20) to rationalize protein hydrogen exchange data in general (21). The protein 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:

$$N \underset{k_2}{\overset{k_1}{\rightleftarrows}} I \xrightarrow{k_3} \text{exchange} \qquad (1)$$

In the I conformations hydrogen exchange can readily take place with a first order rate constant, k_3, whose magnitude approximates the first order exchange rate constants for low molecular weight compounds under similar conditions. In this conformation the labile hydrogen is expected to be hydrogen-bonded to the solvent. Furthermore, it is assumed that the transconformational reactions between the N and I states are characterized by first order rate constants k_1 and k_2.

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's for these equilibria have not been measured. As judged from the acetyl hydroxamate stability constants, alumichrome should be a weaker complex than ferrichrome (22). For ferrichrome, Anderegg et al. (23) 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 (11). 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 realtively faster rates and which can be assigned to the metal-free peptide.

Following the nomenclature of Hvidt and Nielsen (21), the equilibrium between the "native" (the hexadentate chelate) peptide and any and all of its "unfolded" (partially or totally non-chelated) 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 even as the steady-state conformational fluctuations implied in the above equilibrium occur, the degree and kind of exposure that the individual amide NH's need in order to exchange the hydrogen atom through the k_3 process might not be the same. In general, the k_1/k_2 ratios as well as the conformational change contributions to the free energy of activation of the overall 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 minimum at about pH 3 (21,24). Our exchange data for alumichrome shows, however, that in going from pD 5.14 to 3.23 the rates of hydrogen exchange for all the ornithyl amides have increased from exchange half times of 416, 220 and 219 minutes to 12.3, 8.0 and 9.3 minutes for Orn^1, Orn^2 and Orn^3, respectively (Table II). However, the exchange half time for Gly^3 amide does not change much, from 6.2 to 7.5 minutes, indicating a negligible dependence of its exchange kinetics on pD. On the basis of the H^+ concentration dependence of the "unfolding" process discussed above, 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

Table II

for this effect and the overall kinetics are accelerated. For Gly^3 an almost exact compensation results so that no major kinetic change is observed. At about pD 3 important fluctuations around the equilibrium conformation occur and the four slow amides are rather equivalent with respect to exchange. At this pD, stability differences due, e.g., to intramolecular hydrogen-bonding are of no major kinetic difference since the exchange proceeds mainly through the relatively abundant unfolded conformation. At pD 5.14 the chelate is stabilized, the overall ground state conformation is enforced and the conformational fluctuations are of smaller amplitude. At this pD small stabilizing differences such as intramolecular hydrogen bonding will show a more pronounced relative effect. Thus, even though the amide hydrogen of Gly^3 finds itself attached to a more rigid backbone at pD 5.14 than at pD 3.23, it will exchange faster than the similarly located Orn^3 amide hydrogen because the conformational distortion required for it to become exposed does not necessitate the breaking of any significant intramolecular hydrogen bond. As will be seen later, the enthalpy and entropy contributions to the activation free energy support this interpretation.

On raising the pD from 5.14 to 7.22 the stability of the chelate is increased further and the stabilizing effects mentioned above become even more characteristic for each amide. At pD 7.22 intrinsic rates of amide hydrogen exchange due to base catalysis are relatively large. An overall acceleration of the kinetics is observed in alumichrome for Gly^3, Orn^2 and Orn^3, so that their exchange half times drop from 6.2, 220 and 219 minutes to too fast to measure, 9.8 and 2.8 minutes, respectively. A change in pD from 5.14 to 7.22 results in a 20- and 75-fold

increase in H-D exchange rates for the amides of Orn^2 and Orn^3, respectively. This pD change, however, does not appear to affect significantly the exchange rate of Orn^1, whose amide hydrogen exchange half time changes from 416 minutes (pD 5.14) to 362 minutes (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 Gly^3 is about 10^2 larger than for the ornithyl amides and, at neutrality, the order of the observed rate constants differ one from the other by at least one order of magnitude (2.4×10^{-1}, 7.0×10^{-2} and 1.9×10^{-3} min^{-1} for Orn^3, Orn^2 and Orn^1, respectively). This amplification of the kinetic differences among the four amides as the pD is raised from 3.23 to 5.14 to 7.22 is probably a reflection of a change in the hydrogen exchange mechanism with pD. This trend for each of the slow amides is in complete agreement with the stability of the peptide conformation as reflected in the tightness of binding to the metal. At low pD, where the stability is low, the amide hydrogens show first order rate constants of hydrogen exchange which are comparable to those of the conformationally loose poly-D,L-alanine. The expression

$$k_{D_2O} = 50(10^{0.3-pD} + 10^{pD-6.3})10^{0.05(t-20)} min^{-1} \qquad (2)$$

(where t = temperature in °C), proposed by Hvidt and Nielsen (21), yields $k = 0.18$ min^{-1} for this polymer. As the pD is raised, the rate constants for the alumichrome amides depart quite dramatically from the values predicted for poly-D,L-alanine, 6.2 and 741 min^{-1} at pD 5.14 and 7.22, respectively, and remain far below these values.

Since our measurements were made on the hydrogen-deuterium exchange in the aluminum complex, it is of interest to compare these data with

the bulk hydrogen-tritium exchange results obtained by Emery (17) for ferrichrome itself by the Englander two-column gel filtration technique. To facilitate comparison we reproduce Emery's data for the exchange at 30°C together with the half times for alumichrome at the same temperature calculated on the basis of the determined $\Delta H^{\dagger}$ and $\Delta S^{\dagger}$ values reported herein (Table III).

Table I

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 (as would be manifested by coloring of the solution as the Fe^{+3} is coordinated), while it is known that $^{59}Fe^{+3}$ readily exchanges with ferrichrome (about 8 minutes for half completion) at pH 6.3 and 37°C (25). However, these differences in the 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 nitrogens.

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.

Since hydrogen exchange in the presence of excess Fe^{+3} did not affect the kinetics of the process, Emery suggested that the Linderstrøm-Lang mechanism may not apply to the ferrichromes. Excess Fe^{+3} should shift the deferriferrichrome $\rightleftarrows$ ferrichrome equilibrium to the right and hence reduce the availability of "unfolded" peptide concomitantly lowering the rate of hydrogen exchange. On this basis, 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 hydrogen exchange rates. Thus, random modulation of the distance between the metallic center and each sidechain 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, addition of extra metal to the exchanging solution could result in very small effects on the exchange rates as the "local" concentration of Fe^{+3} at the binding center would remain essentially invariant. This would result in the kinetics of the folding process being practically independent of the excess metal ion concentration. Furthermore, and even if the concentration of the metal-free peptide were decreased in the presence of excess metal, this effect would be obscured by the formation of 1:1 complexes, thus opening the molecule. Evidence supporting the formation of a ferric complex of ferrichrome in the presence of excess iron has been reported (26).

In discussing Emery's data for the ferrichrome hydrogen exchange Laiken et al. have suggested the possiblity of a Linderstrøm-Lang EX_1

(unimolecular exchange, vide infra) mechanism for the ferrichromes (8). The relative insensitivity of the ferrichrome hydrogen exchange towards pH changes might, these authors suggest, reflect an insensitivity of k_1 in expression (1) which would be rate-limiting in the Linderstrøm-Lang mechanism. According to the interpretation given above, we believe that it is the relatively strong dependence of k_1 on pH that results in the relative pH independence of the overall exchange rates observed both in the ferrichromes and in the alumichromes.

The components of the activation barrier

Values for $\Delta H^{\dagger}$, $\Delta S^{\dagger}$ and $\Delta F^{\dagger}$ (25°C) were calculated for N-methyl-acetamide on the basis of Eyring plots from the exchange data of Klotz and Frank (27):

	$\Delta H^{\dagger}$ kcal/mole	$\Delta S^{\dagger}$ eu	$\Delta F^{\dagger}$ (25°C) kcal/mole
acid	24	12	20
base	21	5	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 $\Delta H^{\dagger}$ and $\Delta S^{\dagger}$ since the transition state I might require H-bond breakage and even partial unfolding of its secondary and/or tertiary structure.*

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*Woodward and Rosenberg (28,29) have found that native ribonuclease exhibits two classes of amides according to their apparent activation energies for hydrogen-tritium exchange. One class shows activation energies of ∿22 kcal, i.e., similar to that of a number of model compounds which have been studied (21,27,30), while the other class exchanges with activation energies of ∿60 kcal. This latter class is,

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

At pD 5.14, the data (Table II) show that the $\Delta S^{\dagger}$ values are all negative, decreasing in the order Orn^2 (-4.7 eu) > Orn^3 (-5.8 eu) > Orn^1 (-9.3 eu) > Gly^3 (-17.1 eu), which might indicate a trend of decreasing accessibility of the particular hydrogen to exchange. The $\Delta H^{\dagger}$ values show a parallel increase so that at 25°C the three ornithyl amides have about the same free energies of activation ($\Delta F^{\dagger}$ = 23.3 to 23.7 kcal) while the Gly^3 is about 2 kcal ($\Delta F^{\dagger}$ = 21.2 kcal) below these values.

Our kinetic data proves useful in pointing out conformational differences between Gly^3 and Orn^3. Both are symmetrically located within the peptide backbone ring in a sort of anti-parallel β-pleated sheet structure. From the X-ray structure only the Orn^3 amide is transannularly hydrogen-bonded; however, by inspection of

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however, associated with the temperature- or urea-induced unfolding transition of the protein and does not result from the kind of "low amplitude" conformational fluctuation which the I state(s) represent and which we refer to in the text.

atomic models a virtual conformational identity between the two paired amide-carbonyl groupings of Gly^3 - Orn^3 is apparent. For alumichrome in solution, the PMR chemical shifts, their temperature dependences and the gross exchange behavior of its amides did not allow us to discern the relative contributions of hydrogen bonding and steric effects to the stability of these amide hydrogens (11). It is, hence, of interest to compare their heats and entropies of activation. Since $\Delta H^{\dagger}$ for Gly^3 is about 5.5 kcal below the value for Orn^3 (= 20.9 kcal) and hydrogen bond energies are of the order of 3 to 8 kcal/mole, the enthalpy difference between these two amides falls well within the range that contributions due to hydrogen bond stabilization would make. Leichting and Klotz (31) have discussed the importance of inductive effects on the stability of this amide towards exchange resulting from groups on both sides of the -CONH- group. In ferrichrome the Gly^3 - Orn^3 amide pair is similarly located within the ring in the sense that both amides have an ornithyl sidechain to one side and a glycyl α-hydrogen on the other, so that to a first approximation, the inductive effects on the hydrogen exchange of this pair should be similar. However, since the side chains of these two residues are so different, the closer proximity of the ornithyl side chain to the Orn^3 amide NH relative to the Gly^3 NH could result in some detectable exchange rate differences. Indeed, at 25°C the pK_a's of ornithine are 1.94 and 8.65 and of glycine 2.34 and 9.60 (32). 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. This reinforces the suspicion, supported by the differences in $\Delta H^{\dagger}$ for exchange, that while Orn^3 is transannularly hydrogen-bonded, Gly^3 might not be in this state.

Why then the relative stability of the Gly^3 NH towards hydrogen exchange? The answer is given from the $\Delta S^{\dagger}$ differences: the Gly^3 NH is about 11.3 eu (=3.4 kcal at 25°C) more stable than the Orn^3 NH, suggesting that indeed steric effects are relatively more important in shielding the glycyl than its paired ornithyl.

At pD 3.23 and 25°C the $\Delta F^{\dagger}$ values are similar for all the slow amides, ornithines and glycine alike. Such is not the case for the $\Delta H^{\dagger}$ and $\Delta S^{\dagger}$ contributions. The large negative $\Delta S^{\dagger}$ values found for the ornithyl residues probably indicate that their exchange is proceedings through a different conformation, favored by relatively lower $\Delta H^{\dagger}$ values (16.3 kcal $\gtrsim \Delta H^{\dagger} \gtrsim$ 14.5 kcal). The mechanism involves the acid-catalyzed metal exchange which amplifies the conformational fluctuations. It is probable that at this lower pD the $\Delta H^{\dagger}$ is a more reliable reflection of the "intrinsic" hydrogen exchange barrier associated with ornithyl amides since the unfolding mechanism generated by the metal exchange, by being acid-catalyzed, should involve less energy of activation. For Gly^3, the $\Delta H^{\dagger}$ and $\Delta S^{\dagger}$ values for hydrogen exchange at this pD would suggest a different interpretation than that given above for the ornithyl amides. It could be imagined, for example, that the randomization of the ornithyl side chains' conformation is accomplished by a swing toward and away from the metal center while pivoting on the peptide backbone. In such a process the Gly^3 amide hydrogen could be offered a less favorable conformation

for exchange and the process could thus result in larger $\Delta H^{\dagger}$ and smaller $\Delta S^{\dagger}$ values. However, any plausible explanation for the hydrogen exchange of this amide is obscured by the relatively larger errors of its kinetic parameters, reflected in the standard deviations given in Table II. At this lower pD the ornithyl amides provide, hence, a clearer picture of the molecular fluctuations than do the figures for the Gly^3 hydrogen exchange, not only because of a relatively better linear least squares fit of the experimental points, but also because they are a more sensitive probe of the conformational stabilizing effect of metal chelation.

Mechanistic implications

By calculating the exchange rate constant k_3 for a random polypeptide, namely, poly-D,L-alanine, on the basis of equation (2), and dividing the observed k by this number, a gross estimate can be obtained of the relative impedance to exchange for the different amides under the various pD conditions. It is within the framework of the Linderstrøm-Lang, Hvidt and Nielsen treatment that the meaning of $\frac{k}{k_3}$ becomes mechanistically meaningful (21). According to the theory, if the exchangeable amide hydrogens do not remain too long in the exposed state ($k_1 < k_2$), two extreme limits are of interest, the unimolecular EX_1 ($k_1 < k_2 \ll k_3$) and the bimolecular EX_2 ($k_1 < k_2 \gg k_3$) mechanisms. In the EX_1 mechanism the rate limiting step is the unfolding of the native structure and the observed k equals k_1, while in the EX_2 mechanism the leak-out k_3 process is weighted by the extent of unfolding $\left(\frac{k_1}{k_2}\right)$ of the peptide and the observed k equals $\frac{k_1}{k_2} k_3$.

For alumichrome at pD 3.23 and 25°C, $0.3 \lesssim \frac{k}{k_3} \lesssim 0.5$ for the slow amides, which is too large for an EX_2 mechanism. By contrast, the EX_1

mechanism would imply $k = k_1 \ll k_3$ which is contradictory since $k_3 \cong$ 0.2 min^{-1} (poly-D,L-alanine) and the observed $k \cong 0.4$ min^{-1}. It is then likely that at this pD the actual mechanism be intermediate between the extremes EX_1 and EX_2, namely, $k_1 < k_2 \cong k_3$.

From the experimental pD dependence of the hydrogen exchange rate constant above pH 4 for poly-D,L-alanine [equation (2)] it can be derived that

$$\left(\frac{\partial \log k_3}{\partial pD}\right)_T = 1$$

By assuming k_1 and k_2 to be relatively pH independent, it can be shown that an EX_2 mechanism should yield:

$$\left(\frac{\partial \log k}{\partial pH}\right)_T = 1 \quad \text{[Hvidt and Nielsen (21)]}$$

This analysis was applied to alumichrome at pD 5.14 by studying the exchange kinetics at pD 5.04. The data at these two pD's as shown in Table I and Figure 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 Gly^3 rate is halved, i.e.,

$$\left(\frac{\partial \log k}{\partial pD}\right)_{T=25} = \begin{matrix} 1.6 \text{ for } Gly^3 \\ -1.1 \text{ for Orn} \end{matrix}$$

Thus, while Gly^3 seems to satisfy the above criterion and might exchange through an EX_2 mechanism, the data are less clear for the ornithyl amide mechanism. This minor pD shift appears to affect the Gly^3 and Orn values of $\Delta H^\dagger$ and $\Delta S^\dagger$ in different ways. In the case of Gly^3 the decrease in the exchange rate is due to a 6.5 eu drop in $\Delta S^\dagger$ that overcomes the opposing 1.5 kcal drop in $\Delta H^\dagger$. The ornithyl amides, however, decrease

their exchange rate due to slightly more favorable values of both $\Delta H^{\dagger}$ and $\Delta S^{\dagger}$. 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 pD 3.23. By contrast, changes in the Gly^3 amide kinetic parameters, in accordance with the k_3 dependence on pD, are suggestive of a tighter coupling of this amide to a relatively more pD invariant peptide backbone conformation. The $\frac{k}{k_3}$ ratio, however, although larger for Gly^3 than for the ornithyl amides, are all of the right order of magnitude for an EX_2 exchange mechanism at this pD. In summary, it is proposed that the reason why the above partial differential criterion does not apply for the ornithyl amides is that their k_1 and k_2 values are so dependent on the acidity of the medium that they fall outside its range of applicability.

On raising the pD from 5.14 to 7.22, metal chelation-dependent k_1 decreases and k_2 increases. The ratios $\frac{k}{k_3}$ now yield the values 0.36×10^{-5}, 9.54×10^{-5} and 32.9×10^{-5} for Orn^1, Orn^2 and Orn^3, respectively. The range covered is about two orders of magnitude, suggesting now more differentiated exchange pathways within the applicability of an EX_2 mechanism, if it applies to them at all. Indeed, at pD 7.22, $k_3 = 7.4 \times 10^2$ and it is likely that an EX_1 mechanism ($k_3 << k_2 < k_1$) is responsible for the exchange. The relative probabilistic weight of an EX_1 over an EX_2 mechanism increases the greater k_3 and the smaller the $\frac{k}{k_3}$ ratio so that, especially for the case of Orn^1, the EX_1 contribution to the observed kinetics might be relatively important.

For Orn^1, pD 7.22, we find a $\Delta S^{\dagger}$ of about 4 eu/mole. The $\Delta S^{\dagger}$ for base-catalyzed hydrogen-deuterium exchange in N-methyl-acetamide is

estimated to be about 5 eu/mole [from data of Klotz & Frank (27)]. If, due to the high stability of the chelate at this pD, we assume an EX_1 exchange mechanism, then $k \cong k_1$, which implies $\Delta S^\dagger \cong \Delta S_1^\dagger$. There would thus be little or no change in the "ordering" of the system in going through the activated state and hence, presumably, no significant conformational change, as would be required by this mechanism. This excludes the possibility that an EX_1 mechanism be operative. If one assumes that an EX_2 mechanism explains the exchange, and since $k = \frac{k_1}{k_2} k_3$ and $\Delta S^\dagger = 5$ eu/mole for the k_3 process on the basis of the N-methylacetamide data, it is then quite obvious that $\Delta S_1^\dagger - \Delta S_2^\dagger = 0$ and this is indeed very unlikely since the I state, by being unfolded, should have an algebraically larger entropy value than that of the tight chelate, the "native" state. Since none of the proposed mechanisms seems to agree with the experimental data, we support the idea that at neutral pD the exchange proceeds through direct interaction between the exchanging species, i.e., without involving any major unfolding to expose the buried amide. The high enthalpy of activation is easily understood as the barrier for the aqueous hydroxyl to reach the buried Orn^1 NH site should be high. It is likely that the internal amide hydrogens in the alumichromes may serve to illustrate a principle of wider applicability in proteins; the magnitude $\Delta S^\dagger$ should indicate which mechanism is predominant:

$\Delta S^\dagger$	mechanism
large	unfolding
small	direct

Leichtling and Klotz (31) and Klotz and Mueller (33) have presented good evidence that the local environment surrounding the exchanging amide has

definite influence on its hydrogen exchange behavior. Klotz (34) has distinguished between intrinsic and extrinsic factors regulating the exchange kinetics. The intrinsic factors are to be found in the amide itself, namely, hydrogen-bonding, -CONH- neighbor group inductive effects, etc. Extrinsic factors will depend on the solvent (polarity, composition, pH, etc.) and the local environment (hydrophobic or hydrophilic character). According to the Berger, Loewenstein and Meiboom mechanism (35), the base-catalyzed hydrogen exchange involves the existence of an anionic intermediate. Hence, the Orn^1 amide, by finding itself buried in a highly non-polar surrounding, would show a reduced rate of hydrogen exchange due to a relative de-stabilization of the anionic intermediate by its hydrophobic environment. This effect would then add to any stabilization of the starting ground state, the hydrogenated amide, due, for example, to mere steric protection.

At pD 7.22 the analysis of the Orn^1 amide hydrogen exchange agrees with a relatively rigid picture of the peptide conformation as regulated by the side chain chelation to the metal. As previously indicated, however, the extent of the unfolding required by all the amides to proceed through a Linderstrøm-Lang mechanism might be different, so that at neutrality enough conformational flexibility might be present to allow the Orn^2 and Orn^3 amides to follow this mechanism even if the more buried Orn^1 does not. As has been pointed out by Emery (17), ferrichrome lacks any free charged group whose titration could result in drastic conformational transitions. One would expect that any conformational fluctuations present at pD 7 will also be present at lower pD, in addition to those controlled by the metal. The $\Delta S^{\dagger}$ values for Orn^2 and Orn^3 amide hydrogen exchange

at pD 7.22 show that these extra contributions to the conformational fluctuations, if present, 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.

The term "motility" has been used to indicate rates of folding-unfolding ($k_1 + k_2$) of protein molecules. Originally EX_1 and EX_2 exchange mechanisms were implied as being of low and high motility respectively. Even though this correspondence is not rigorous [see (21), p. 319], it still has some heuristic value in that it gives an idea of the frequency of the conformational fluctuations. An interesting feature of the alumichrome data is that within the range $3 \lesssim pD \lesssim 7$ they suggest little variation of the net "motility" ($= k_1 + k_2$) even though the $\frac{k_1}{k_2}$ ratio might vary through several orders of magnitude. Alumichrome, hence, exemplifies the relevance of the relative weights of the folded to the unfolded state, rather than the absolute conformational fluctuation frequency, as a major determinant of the hydrogen exchange mechanism.

Gramicidin S-A vs. alumichrome

Linear interpolation of the reported (8) gramicidin S-A 0°C rate constants to pH 3.23 yields a half life of hydrogen exchange of about 2,500 minutes ($k = 2.8 \times 10^{-4}$ min^{-1}). If one calculates these same kinetic parameters for the slow amide hydrogens of alumichrome at 0°C from the $\Delta H^{\dagger}$ and $\Delta S^{\dagger}$ values at pD 3.23 (Table III), the figures for the half lives and rate constants are:

	Gly^3	Orn^1	Orn^2	Orn^3
$t_{1/2}$ (min)	180	126	98	126
k (min^{-1})	3.8×10^{-3}	5.5×10^{-3}	7.1×10^{-3}	5.5×10^{-3}

For poly-D,L-alanine (free amides) the exchange rates calculated for these same conditions yield half lives of ~68 minutes ($k \sim 10^{-2}$ min^{-1}). It is then clear that although some kinetic stabilization of alumichrome relative to poly-D,L-alanine might be present, under these same conditions gramicidin S-A would appear to have a much more rigid conformation, the difference in hydrogen exchange $\Delta F^{\dagger}$ being about 1.2 kcal. An explanation for this must be sought in the different mechanisms through which these two peptides exchange. While the unfolding in alumichrome is acid-catalyzed, the conformation of gramicidin should be essentially insensitive to pH.

In alumichrome the reduced rate of amide hydrogen exchange, even at pD 3.14 and 0°C could still be accounted for in terms of the relative conformational rigidity enforced on the peptide by the metal chelation. Since in gramicidin S-A this stabilizing force is absent, we considered it convenient, for comparative purposes, to observe the exchange kinetics of the metal-free peptide. The PMR spectrum of the deferriferrichrome amide NH region, in water, shows on going from low to higher fields, first a triplet, corresponding to a single glycine amide (Gly_1), then a broader complex band which is the overlap of Gly_2 + Gly_3 + Orn_1, and finally, two separate doublets, corresponding to Orn_2 and Orn_3 (11). The spectrum at 23.5°C shows lines that are slightly broader than at 58°C. Due to this broadening, at lower temperatures the last two ornithyl peaks are not well resolved. Hence, the exchange of these three resolved bands was followed at pD 3.0 and 3.5°C. The measured half lives and rate constants are:

	band 1 (Gly_1)	band 2 (Gly_2+Gly_3+Orn_1)	band 3 (Orn_2+Orn_3)	poly-D,L-alanine
$t_{1/2}$ (min)	62±2	80±2	84±2	19
k (min^{-1})	$(11.2\pm0.4)\times10^{-3}$	$(8.6\pm0.2)\times10^{-3}$	$(8.2\pm0.2)\times10^{-3}$	36.6×10^{-3}

The values for poly-D,L-alanine were calculated as before. Although the data suggests a more protected location for the amides with resonances at higher fields this protection is not great relative either to the calculated values of random poly-D,L-alanine or even less to the values reported for gramicidin S-A under similar conditions. 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 differentiation between PMR observable amide hydrogens in synthetic cyclic hexapeptides (36,37).

In the previous discussion the hydrogen exchange kinetics of deferriferrichrome, alumichrome and gramicidin S-A were compared at about pH 3. At higher pH's, however, 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 consequence of Klotz's approach to the theory of hydrogen exchange is that even though base-catalyzed rates should be dependent on hydroxyl ion concentration, the activation energies will remain constant (27). The alumichromes do not satisfy this rule. Relatively large changes in the $\Delta H^{\dagger}$ and $\Delta S^{\dagger}$ values resulted for each single amide as the pD was varied. Since the PMR spectrum of the amide region does not change, as

would be expected for a pD-driven conformational drift, the local (N state) milieu around each amide remains constant with pD so that environmental changes cannot account for the observed variations in the kinetic parameters.

By contrast, we have noted the inadequacy of either an EX_1 or an EX_2 Linderstrøm-Lang mechanism to account for the observed hydrogen exchange rates of Orn^1 at pD 7.22. It was suggested that poor direct accessibility of this particular amide to the solvent plus certain de-stabilization of the anionic intermediate by its hydrophobic environment could account for its observed low rate of exchange.

A summary of our view of the pD effect on the mechanism for the alumichrome amide hydrogen-deuterium exchange is diagrammatically presented in Figure 5. Within the Linderstrøm-Lang hypothesis there is first a free energy barrier ($\Delta F_1^\dagger$) to reach the intermediate, unfolded state I, followed by a second free energy barrier ($\Delta F_3^\dagger$) for the direct H-D exchange of the unhindered amide. While $\Delta F_1^\dagger$ has a relatively high entropic contribution, $\Delta F_3^\dagger$ is mainly enthalpic. In our experiments on alumichrome OD^- catalyzes the second step while D^+ catalyzes the first. In Figure 5 the relative trends of the $\Delta F_1^\dagger$ and the $\Delta F_3^\dagger$ barriers with pD are depicted. At pD $\tilde{\sim}$ 3 the $\Delta F_1^\dagger$ barrier is so low that the overall exchange rate is determined by the $\Delta F_3^\dagger$ step, the intrinsic rate of amide hydrogen-deuterium exchange being minimum at about this pD. At high pD ($\sim$7) the structure becomes reinforced because of the high kinetic stability of the chelate moiety. Even though the intrinsic amide exchange barrier, $\Delta F_3^\dagger$, is now relatively low due to the base catalysis, the height of the $\Delta F_1^\dagger$ barrier results in measurably low exchange rates. At neutrality, and at least for the more buried Orn^1 amide, it is very likely that the

exchange does not proceed to a significant extent through a Linderstrøm-Lang mechanism. The relatively more extensiye conformational change which would be required for this amide to become exposed suggests that its exchange might instead proceed by direct reaction with the solvent. Thus, in the folded N conformation, its exchange rate would be highly reduced by its hydrophobic environment as well as by its steric inaccessibility. 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 Al^{+3} trihydroxamate complex than at lower pD and some exchange retardation becomes apparent. Although at this pD the Linderstrøm-Lang mechanism may still be predominant, the direct exchange, without unfolding, may commence to contribute to the measured rates.

A merit of alumichrome as a model for amide hydrogen exchange is that it clearly shows the relative requirement of both the Klotz and the Linderstrøm-Lang approaches for explaining the observed exchange kinetics. Both local environmental effects and conformational fluctuations are present: while the second dominates the exchange rates around pD 3, the first becomes dominant around neutrality, with a gradual transition of one mechanism into the other on passing through intermediate pD's. The relative contributions of each mechanism depend on the particular amide and its location within the molecule.

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

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Table I

Alumichrome

T	Gly^3	Orn^1	Orn^2	Orn^3
		(a) pD 3.23		
4.7	0.0041±0.0002			
9.6	0.0207±0.0009	0.0164±0.0007	0.0202±0.0011	0.0190±0.0011
12.8	0.0300±0.0023	0.0184±0.0027	0.0332±0.0004	0.0210±0.0018
17.3	0.0358±0.0011	0.0243±0.0011	0.0305±0.0006	0.0291±0.0008
24.2	0.0761±0.0016	0.0536±0.0026	0.0797±0.0025	0.0723±0.0022
29.5	0.1377±0.0038	0.0660±0.0047	0.1276±0.0073	0.1096±0.0040
34.0	0.2455±0.0135	0.1470±0.0077	0.2186±0.0172	0.1917±0.0181
		(b) pD 5.04		
17.3	0.0282±0.0004	0.0010±0.0001	0.0019±0.0001	0.0012±0.0001
24.2	0.0480±0.0011	0.0030±0.0001	0.0053±0.0001	0.0036±0.0000
30.5	0.0675±0.0017	0.0081±0.0003	0.0128±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±0.0082	0.0180±0.0005	0.0377±0.0016	0.0230±0.0007
51.0	0.4029±0.0169	0.0536±0.0010	0.1113±0.0053	0.0744±0.0064
		(c) pD 5.14		
17.3	0.0557±0.0013	0.0005±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	0.0034±0.0001	0.0062±0.0001	0.0062±0.0001
34.0	0.2693±0.0119	0.0044±0.0001	0.0094±0.0001	0.0102±0.0002
41.2	0.4792±0.0315	0.0104±0.0009	0.0217±0.0014	0.0204±0.0011
51.0		0.0316±0.0015	0.0666±0.0022	0.0632±0.0029

Table I (Cont.)

T	Gly^3	Orn^1	Orn^2	Orn^3
		(d) pD 7.22		
5.0			0.0066 ± 0.0001	0.0197 ± 0.0004
9.6			0.0083 ± 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	0.0576 ± 0.0017	0.2422 ± 0.0053
30.5		0.0040 ± 0.0001	0.1065 ± 0.0035	0.3921 ± 0.0179
34.0		0.0078 ± 0.0002	0.2184 ± 0.0064	0.6671 ± 0.0320
41.2		0.0179 ± 0.0005	0.4499 ± 0.0226	
51.0		0.0540 ± 0.0033		

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 as in Fig. 1. The k values together with their standard deviations are tabulated vs. temperature (T) at each pD. The pDs studied were 3.23 (a), 5.04 (b), 5.14 (c) and 7.22 (d). The units in this table 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. The statistical quality of the first order adjustments is reflected in their corresponding standard deviations. 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).

Table II

Alumichrome

	$\Delta H^{\dagger}$	$\Delta S^{\dagger}$	$\Delta F^{\dagger}(25)$	k(25)	$t_{1/2}(25)$
			(a) pD 3.23		
Gly^3	20.0±2.3	-4.4±7.8	21.3±3.2	0.0918	7.55
Orn^1	14.5±1.6	-23.8±5.3	21.6±2.2	0.0564	12.30
Orn^2	15.6±1.7	-19.1±5.9	21.3±2.5	0.0864	8.02
Orn^3	16.3±1.2	-17.2±4.1	21.4±1.7	0.0027	9.31
			(b) pD 5.04		
Gly^3	14.5±0.8	-23.9±2.6	21.6±1.1	0.0511	13.57
Orn^1	20.6±1.4	-9.0±4.5	23.3±1.9	0.0031	224.38
Orn^2	21.6±0.6	-4.5±2.1	22.9±0.9	0.0056	121.88
Orn^3	21.4±1.0	-6.0±3.4	23.2±1.4	0.0038	179.96
			(c) pD 5.14		
Gly^3	16.1±0.6	-17.1±1.9	21.2±0.8	0.1109	6.25
Orn^1	20.9±1.4	-9.3±4.7	23.7±2.0	0.0017	415.87
Orn^2	21.9±0.6	-4.7±2.1	23.3±0.9	0.0031	220.05
Orn^3	21.6±0.8	-5.8±2.6	23.3±1.1	0.0032	219.46
			(d) pD 7.22		
Gly^3					
Orn^1	24.8±0.7	4.2±2.5	23.6±1.0	0.0019	361.79
Orn^2	19.9±1.0	-5.2±3.3	21.4±1.4	0.0706	9.82
Orn^3	20.3±1.2	-1.4±4.0	20.7±1.7	0.2445	2.83

Table II (Cont.)

The activation enthalpy $\Delta H^{\dagger}$ and the activation entropy $\Delta S^{\dagger}$ for the amide hydrogen-deuterium exchange of alumichrome at pD 3.23 (a), 5.04 (b), 5.14 (c) and 7.22 (d). The kinetic parameters correspond to the slowly exchanging amides and have been determined from weighted least squares fits of the absolute reaction rate theory expressions for the temperature dependence of the first order rate constants, given in Table I, and as depicted in Fig. 4. The standard deviations are included, and they reflect the statistical quality of the adjustments. Also included in the table are the activation free energies $\Delta F^{\dagger}(25)$ at 25°C, calculated from the tabulated $\Delta H^{\dagger}$ and $\Delta S^{\dagger}$ on the basis of the expression $\Delta F^{\dagger} = \Delta H^{\dagger} - T\Delta S^{\dagger}$, with their uncertainties as propagated from the standard deviations of $\Delta H^{\dagger}$ and $\Delta S^{\dagger}$. The first order rate constants k(25) and exchange half times $t_{1/2}(25)$, calculated for all the measured amides at 25°C from $\Delta F^{\dagger}(25)$ and the absolute reaction rate theory expression $k = \frac{KT}{h} e^{\frac{\Delta F^{\dagger}}{RT}}$ are also included. These values are convenient since they enable comparison of the relative exchange kinetic behavior of all the amides under identical temperature (25°C) conditions. The reader is reminded, however, that the measured rate constants are given in Table I, together with their experimental uncertainties. As stated in Table I and Fig. 4, the Gly^3 amide exchanges too fast at pD 7.22 and hence its corresponding kinetic parameters are not included in (d). $\Delta H^{\dagger}$ and $\Delta F^{\dagger}(25)$ are expressed in kilocalories/mole, $\Delta S^{\dagger}$ in eu (= cal/(°Kelvin x mole)), k(25) in minutes^{-1} and $t_{1/2}(25)$ in minutes.

Table III

	pH =		3.0	4.7	7.0
(A)	Ferrichrome		48 (3.8)	94 (2.7)	14 (1.8)
	pD =		3.23	5.04	5.14	7.22
(B)	Alumichrome	Gly^3	4.26	8.91	3.93	
		Orn^1	8.08	124.30	228.57	178.18
		Orn^2	5.10	65.68	117.69	5.55
		Orn^3	8.08	97.63	118.42	1.58

Half times, in minutes, of hydrogen-tritium exchange for ferrichrome (A) and of hydrogen-deuterium exchange for its Al^{+3} analogue (B). The values for the ferric complexes were measured at 30°C by Emery (17), while those for alumichrome are calculated, for the same temperature, from the free energy of activation at 30°C and on the basis of the absolute reaction rate theory from the kinetic data ($\Delta H^{\dagger}$ and $\Delta S^{\dagger}$) reported in Table II. The values in parentheses are the number of slowly exchanging hydrogens (per mole of peptide) obtained by Emery by zero time extrapolation of the bulk exchange kinetic curves.

FIGURE CAPTIONS

Fig. 1. The conformational model for ferrichrome (11). Chemical bonds along the peptide backbone ring are represented with heavier lines, and the corresponding residues, whether glycyl or ornithyl, are labeled by numbers in circles at the α-carbon atom. The metal is represented by M and, for simplicity, only those hydrogen atoms belonging to the four slowly exchanging amides, namely, glycyl 3 and the three ornithyls, are shown. While the existence of the hydrogen bond bridging Gly^3-NH··O=C-Orn^3 is not clear from previous work, in this communication it is suggested that in alumichrome the stability of this amide towards hydrogen exchange is mainly due to steric rather than to H-bonding protection. A distinction is made between very weak (···) and stronger (---) hydrogen bonds.

Fig. 2. The 220 MHz amide NH resonance region of 125 mM aqueous alumichrome at 56.5°C. This spectrum was recorded at pH 5.14, but the chemical shifts are invariant within the pH (pD) range studied in this work. Each resonance is assigned to its corresponding residue according to the convention followed in Fig. 1. The chemical shift scale is referred to internal tert-butyl alcohol and is expressed in parts per million (ppm) at the top and in Hertz (Hz) at the bottom.

Fig. 3. Hydrogen-deuterium exchange kinetics for the four slowly exchanging amides in alumichrome at pD 5.14 (0.005 M d_3-acetate) in D_2O and at 24.2°C. The dots plot the logarithm of the experimentally measured proton resonance peak amplitudes vs. time in minutes. The lines are least squares fits of the data points assuming a first order decay

FIGURE CAPTIONS (Cont.)

and, together with the data points, are normalized to 10 for zero time. The experimental points for each amide are marked according to the key included in the figure, which also shows, in parentheses, the times in minutes for half decay of the corresponding amide resonances. The indices identify the resonances according to the convention followed in the text. The exchange rate constants determined from linear fits of this kind, together with their standard deviations, are given in Table I for the slow amides of alumichrome under different pD and temperature conditions.

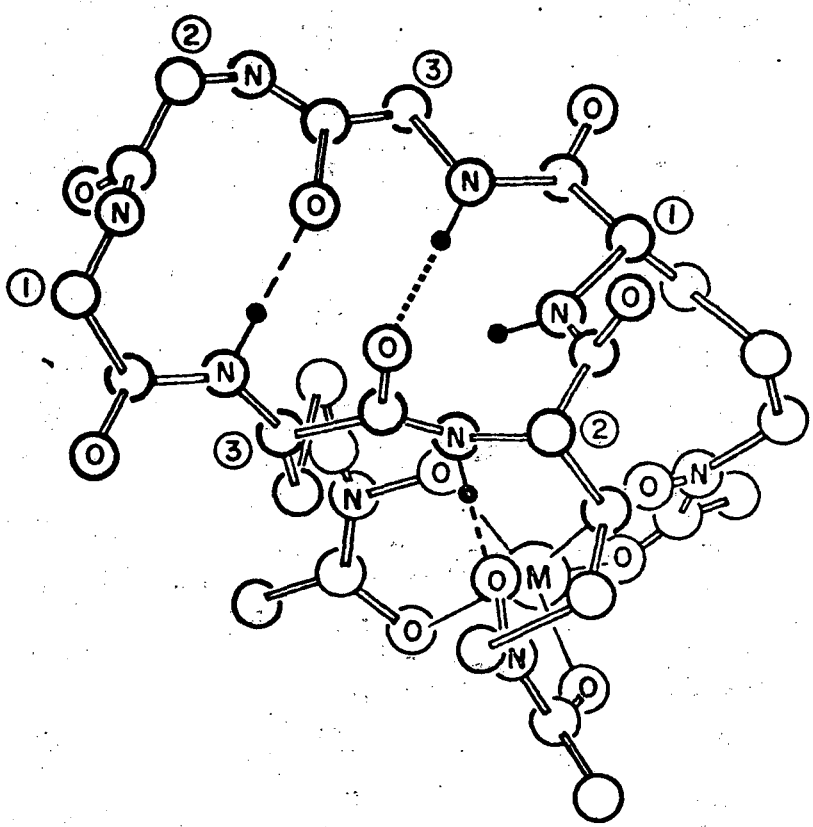
Fig. 4. Absolute reaction rate theory plots for the alumichrome Gly^3, Orn^1, Orn^2 and Orn^3 amide hydrogen-deuterium exchange kinetics at pD 3.23 (a), 5.04 (b), 5.14 (c), and 7.22 (d). At pD 7.22, Gly^3 exchanges too fast to be monitored by this technique. The lines are weighted linear least squares fits of $-R\ln(\frac{h}{KT}k)$ in cal/(°K x mole) vs. 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. 1. The slopes of these linear plots yield $\Delta H^{\dagger}$, the enthalpy of activation, while the intercept (at $T^{-1} = 0$) yields $\Delta S^{\dagger}$, the entropy of activation. $\Delta H^{\dagger}$ and $\Delta S^{\dagger}$ 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.

FIGURE CAPTIONS (Cont.)

Fig. 5. Diagrammatic representation of the pD dependence of $\Delta F^{\dagger}$ of hydrogen-deuterium exchange for a "buried" amide hydrogen (e.g., Orn^{1}) in the alumichromes. The diagram is constructed on the basis of the Linderstrøm-Lang scheme:

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

Vertical scales are arbitrary. The intention here is to show the shifts of $\Delta F_1^{\dagger}$ relative to $\Delta F_3^{\dagger}$ as the pD is varied. It should be noticed that due to the opposite pD dependences which $\Delta F_1^{\dagger}$ and $\Delta F_3^{\dagger}$ exhibit, the net observed $\Delta F^{\dagger}$ is not much affected by pD. In the text arguments are given that support the predominance of this mechanism at pD 3 and even at pD 5, but, for certain amides, not at neutrality.



XBL 701-5020

Fig. 1

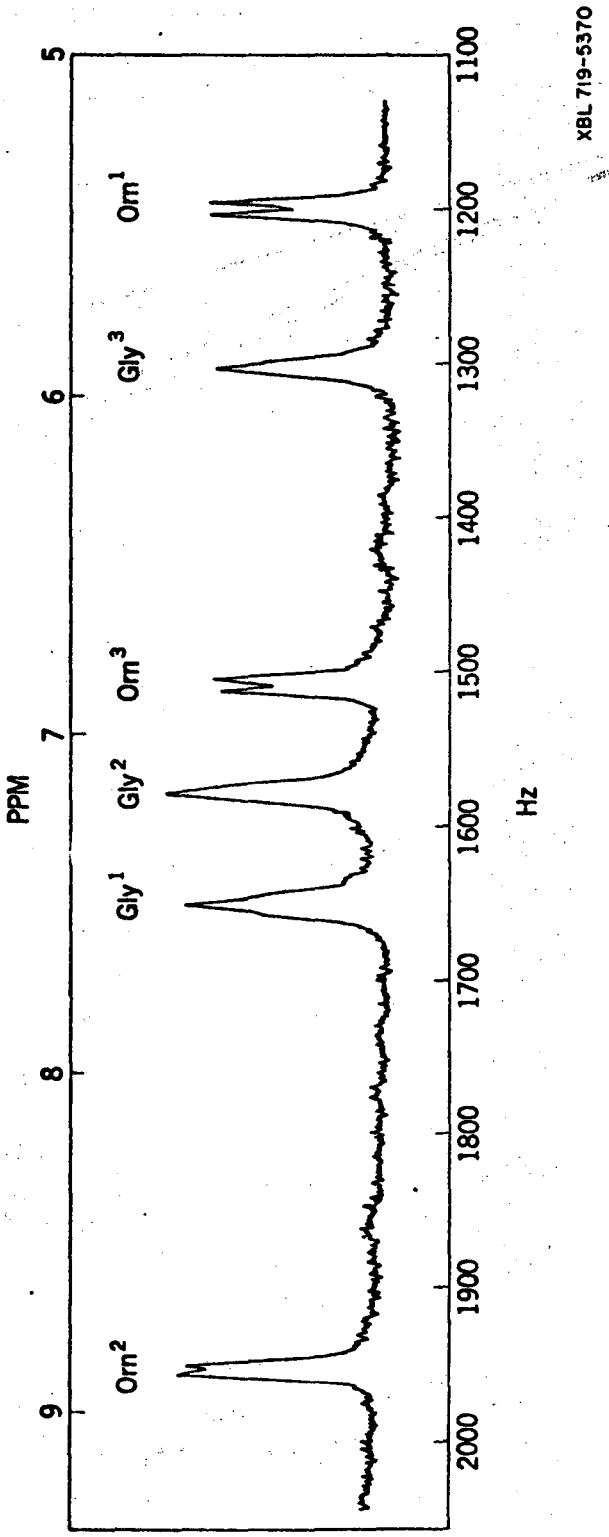

PPM
5
6
7
8
9
Hz
1100
1200
1300
1400
1500
1600
1700
1800
1900
2000
Orn¹
Gly³
Orn³
Gly²
Gly¹
Orn²
XBL 719-5370

Fig. 2

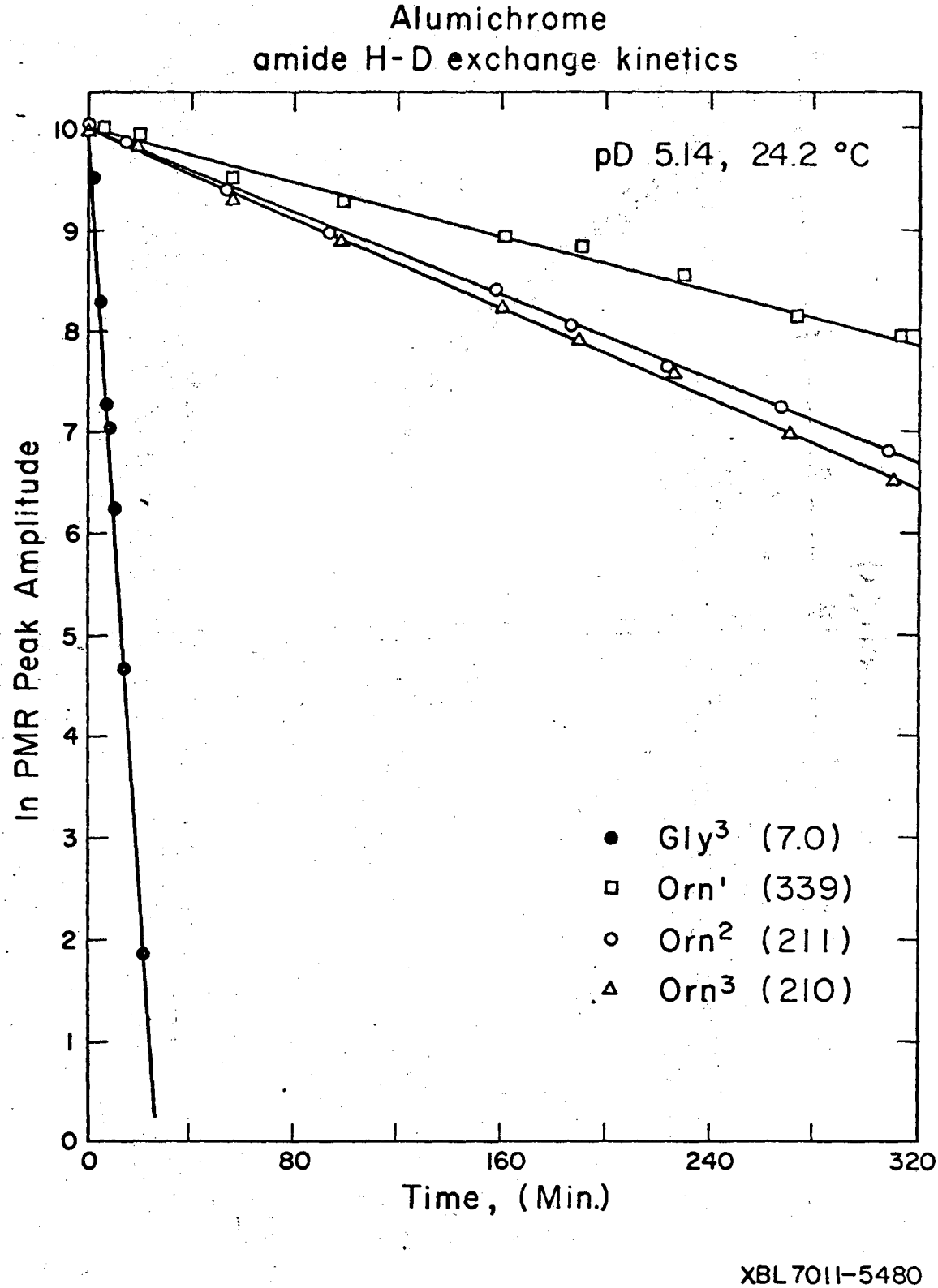

Alumichrome
amide H-D exchange kinetics
pD 5.14, 24.2 °C
ln PMR Peak Amplitude
Time, (Min.)
0
80
160
240
320
0
1
2
3
4
5
6
7
8
9
10
Gly3 (7.0)
Orn' (339)
Orn2 (211)
Orn3 (210)
XBL 7011-5480

Fig. 3

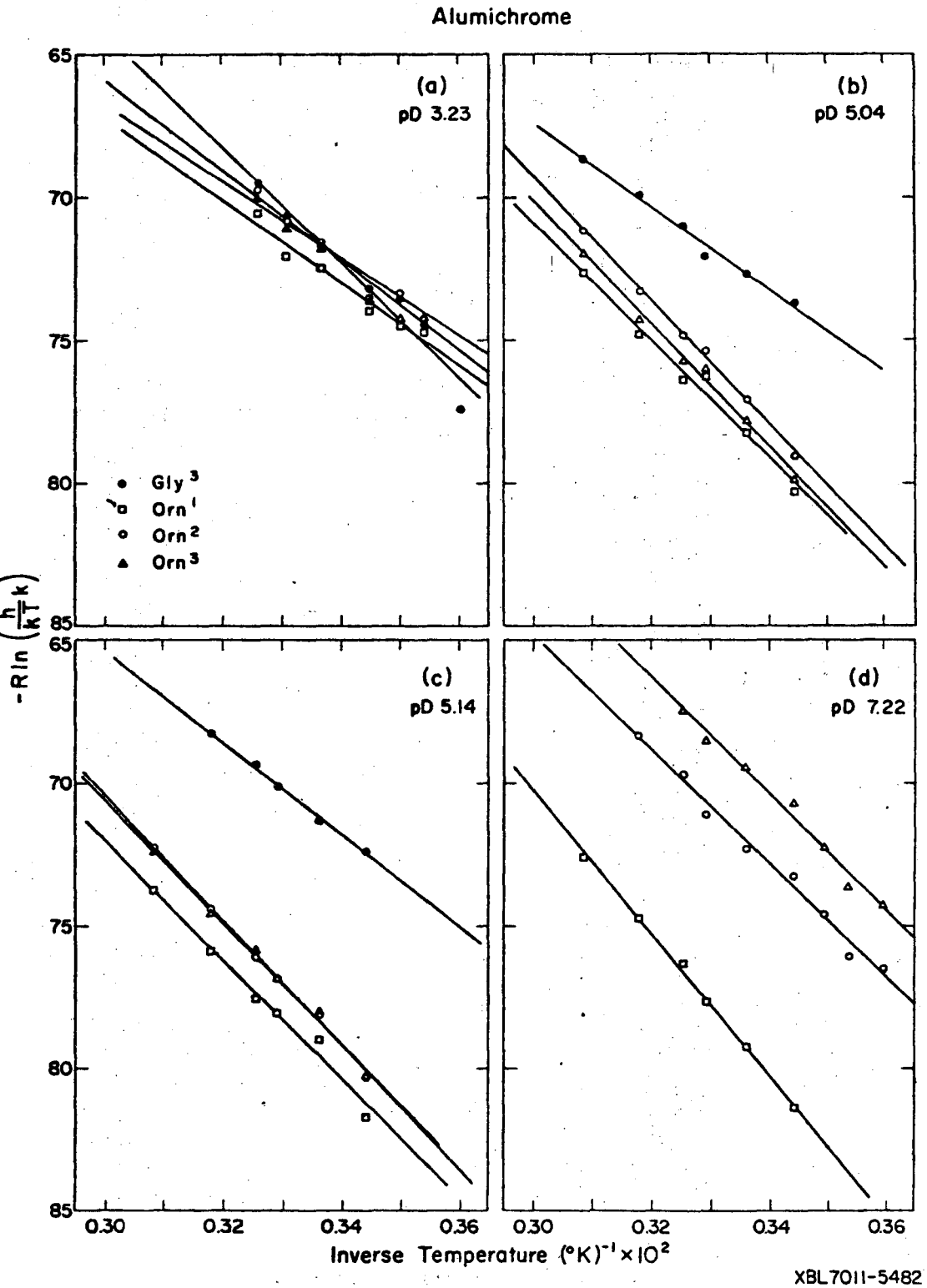
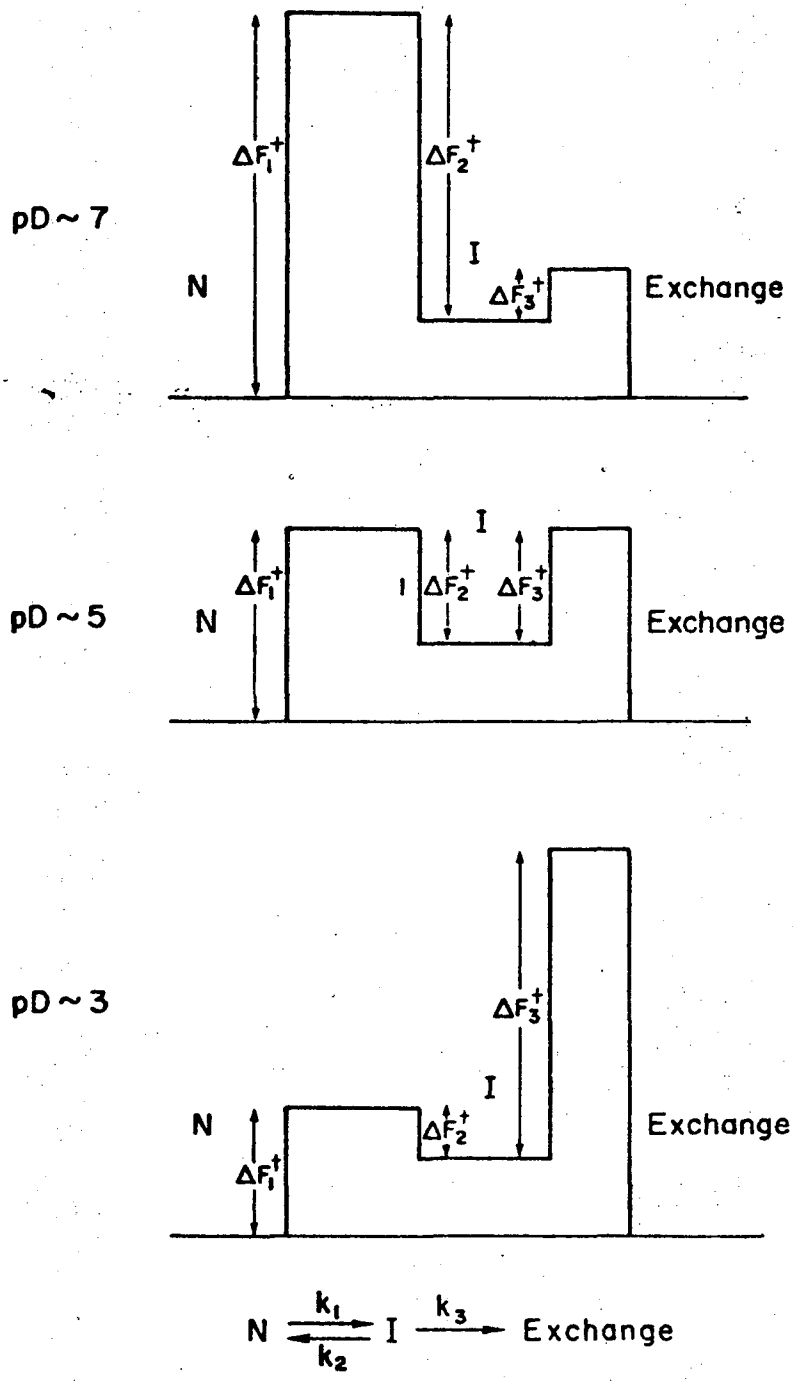

Alumichrome
(a)
pD 3.23
(b)
pD 5.04
(c)
pD 5.14
(d)
pD 7.22
Gly 3
Orn 1
Orn 2
Orn 3
-R ln (h/kT k)
65
70
75
80
85
0.30
0.32
0.34
0.36
Inverse Temperature (°K)⁻¹ × 10²
XBL7011-5482

Fig. 4

The Linderstrøm-Lang hydrogen exchange scheme for the Alumichromes



XBL7011-5479

Fig. 5

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