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Hydrogen–Deuterium Exchange Kinetics of the Amide Protons of Oxytocin Studied by Nuclear Magnetic Resonance†

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ABSTRACT: The contribution of intramolecular hydrogen bonding to the solution structure of oxytocin was evaluated by study of amide hydrogen exchange rates in D_2O by Fourier transform ^1H NMR spectroscopy. Resolution enhancement filtering was employed in the determination of individual pseudo-first-order rate constants. Apparent barriers to exchange of 0.5 and 0.6 kcal mol^{-1} were measured for Asn^5 and Cys^6 peptide NH, respectively. The slowing is best explained by steric hindrance to solvent access in the case of Asn^5, while for the Cys^6 participation in a weak intramolecular hydrogen bond is possible. Fourfold acceleration of base-catalyzed exchange was observed for Tyr^2 NH; it is proposed that this is the result of electronic effects induced by hydrogen bonding of Cys^1 C=O, either to Cys^6 NH or to the N-terminal amino group. Exchange proceeds near the random coil limit for each of the remaining residues. Comparison with exchange data for the model tripeptide *N*-acetyl-L-prolyl-L-leucylglycinamide demonstrates no evidence of noncovalent association of the tocin ring with the tripeptide tail of the hormone.

Elucidation of the solution structure of the neurohypophseal hormone oxytocin [*cyclo*-(H-Cys^1-Tyr^2-Ile^3-Gln^4-Asn^5-Cys^6)-Pro^7-Leu^8-Gly^9-NH_2] (Figure 1) is essential for the understanding of mechanisms of receptor recognition and activation at the molecular level. It has been proposed (Urry & Walter, 1971) that in dimethyl sulfoxide (Me_2SO)[1] the predominant conformers contain two $1 \rightarrow 4$ β turns stabilized by two or more intramolecular hydrogen bonds. Whereas research into the conformation in aqueous solution has yielded much information concerning hydrodynamic properties (Craig et al., 1964), segmental mobility (Glasel et al., 1973; Deslauriers et al., 1974a; Live et al., 1979), rotational isomerism (Wyssbrod et al., 1977; Meraldi et al., 1977), and selected torsional angles (Fischman et al., 1980; Tu et al., 1978; Beychok & Breslow, 1968; Cowburn et al., 1978, 1980), the role of intramolecular hydrogen bonding remains unclear. NMR data have generally been interpreted (Glickson, 1975) to exclude such interactions in aqueous solution, but β-turn structure has been inferred from laser Raman spectra (Tu et al., 1978). Saturation transfer experiments suggest that transannular hydrogen bonds believed to exist in Me_2SO obtain in water as well (Glickson et al., 1976; Krishna et al., 1979). Conformational energy calculations (Kotelchuk et al., 1972; Honig et al., 1973; Nikiforovich et al., 1979) have not proved definitive.

The study of hydrogen-exchange kinetics have been employed extensively as a probe of protein structure (Woodward & Hilton, 1979) and provides a direct approach to the determination of the conformational state of the NH protons. It has been shown (Molday et al., 1972) that in water the peptidyl hydrogens of random coil polypeptides exchange with solvent protons at rates that can be predicted accurately from the amino acid sequence; departure from random-coil kinetics is attributable to the local effects of secondary and tertiary structure. The complete assignment of the amide resonances of the ^1H NMR spectrum of oxytocin (Brewster & Hruby, 1973) thus makes possible the acquisition of conformation-sensitive data for a large number of specific sites in the molecule.

In the present investigation, hydrogen–deuterium exchange (HDX) rates were obtained for oxytocin and *N*-acetyl-L-prolyl-L-leucylglycinamide (NPLG) by monitoring of the proton signals in D_2O by pulse–Fourier transform NMR. Difficulties posed by resonance overlaps were surmounted by use of resolution enhancement filtering (Ernst, 1966; Wittbold et al., 1980) and computer-assisted spectral simulation. The fitted exchange parameters were compared with projections based on the exchange kinetics of the random-coil homopolypeptide poly(DL-alanine) (PDLA) (Englander & Poulsen, 1969; Bryan & Nielsen, 1960) and the conformational significance of any disparities considered in light of the accepted mechanism of hydrogen exchange in amides (Berger et al., 1959; Molday & Kallen, 1972) and structural data obtained by other methods.

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[1] Abbreviations used: HDX, hydrogen–deuterium exchange; NPLG, *N*-acetylprolylleucylglycinamide; NMR, nuclear magnetic resonance; PDLA, poly(DL-alanine); FID, free-induction decay; Me_2SO, dimethyl sulfoxide; TLC, thin-layer chromatography.

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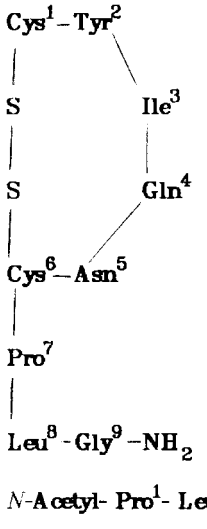

FIGURE 1: The chemical structures of oxytocin and NPLG.

Materials and Methods

Preparation of Materials. Oxytocin acetate was prepared and isolated according to published procedures (Live et al., 1977), employing solid-phase synthetic methods. The *S-p*-methoxybenzyl protecting group was selected for cysteine because of its high degree of lability in anhydrous HF. Benzhydrylamine hydrochloride resin, obtained from Beckman Instruments, Inc., was determined to have 0.43 mequiv of nitrogen/g of resin available for coupling. *tert*-Butyloxycarbonyl-L-amino acids, dicyclohexylcarbodiimide, and 1-hydroxybenzotriazole hydrate were obtained from Chemical Dynamics Corp. and Bachem, Inc. Picric acid monitoring of coupling efficiency gave results comparable to those obtained previously. All analytical results (TLC, NMR, amino acid analysis) were comparable to those previously obtained (Live et al., 1977). The product yield was 48%, based on the number of attachment sites initially present on the resin.

N-Acetyl-L-prolyl-L-leucylglycinamide hemihydrate (NPLG) was synthesized from L-prolyl-L-leucylglycinamide (Chemical Dynamics Corp.) and acetic anhydride. The yield was 87%. Anal. Calcd for $C_{15}H_{26}O_4N_4 \cdot 0.5H_2O$: C, 53.7; H, 8.1; N, 16.7; O, 21.5. Found: C, 53.8; H, 8.0; N, 16.6; O, 21.6. Poly(DL-alanine) (PDLA) of mean chain length 23 residues was the generous gift of Professor S. W. Englander.

Data Acquisition. ^{1}H NMR spectra were obtained on a 220-MHz Varian HR/Nicolet Technology Corp. TT-220 spectrometer by pulse and fast Fourier transform techniques. Sample temperature was measured by using a methanol standard and was 19.5 °C for all exchange experiments. Sample concentrations (w/v) were as follows: oxytocin, 8%; NPLG, 5%; PDLA, 1%. The high concentration of oxytocin was required in order to obtain sufficient signal to noise for resolution enhancement. Chemical shifts are reported with respect to sodium (trimethylsilyl)propionate. The internal standard was added to the control samples and not to those actually used in the kinetic studies.

Standard titration curves for the various samples were measured in order to avoid excessive manipulations and additions immediately prior to the kinetic runs. For each kinetic run, an appropriate amount of acid was added just prior to the run, and the actual pD (glass electrode reading plus 0.40) (Glasoe & Long, 1960) was determined after the data acquisition. Runs made were distributed over the pD range 1.90–4.35.

HDX runs were performed as follows. Balance and field homogeneity were adjusted by using a peptide solution identical in concentration with the exchange samples. The spectrometer was then placed in Fourier transform mode. For initiation of each exchange run, the sample tube was removed from the probe, and its contents were siphoned. Appropriate quantities of sample and dilute DCl in D_2O were mixed rapidly and forced through a fiberglass filter into the same sample tube; this was done to assure removal of any particular contaminants and small air bubbles released during dissolution, either of which could cause loss of resolution in the early portion of the run. The tube was inserted into the probe, fine shimming performed with the audio monitor, and data acquisition begun. The interval from the start of exchange to the start of data acquisition ranged from 40 to 75 s. Each summed free-induction decay (FID) obtained represented the average of between 16 and 24 transients; radiofrequency pulses were of 40-μs duration, 1.648 s apart.

This pulse length and interpulse delay are such that there is an ~99% return to equilibrium between pulses, assuming a T_1 for the amide protons of 0.4 s. There was a delay of 0.5 s between the end of the last acquisition of one FID and the start of the next, for storage of the summed FID on disk. A total of 12–16 such cycles were completed per exchange run.

Processing of Spectra. Prior to Fourier transformation, each FID was convoluted with an exponential decay with a time constant of 0.3 Hz. In the resulting transformed spectrum, the signal to noise ratio was improved by a factor of 2, amide line widths were broadened by ~8%, and the line shape was unaffected. Each 2500-Hz spectrum then occupied 4096 memory locations. Peak amplitudes and positions were calculated from a three-point parabolic interpolation routine.

The kinetic constant R (Table I) was calculated by linear least-squares regression of the logarithmically transformed signal against time. Peak amplitudes were used for oxytocin and NPLG, since there was no variation in line widths within a given exchange run. In a few cases, poor resolution was evident in the first spectrum, and calculations excluded that spectrum.

The presence of superimposed signals presents particular difficulties in kinetic experiments. It is necessary for two rates to be appreciably different to permit their accurate isolation by the simple fit of two time constants to a single set of decreasing values; the problem is especially acute when this set is small. We therefore attempted the simulation of complex multiplets by use of resolution enhancement filtering and iterative methods. This was required for the Asn^5/Gly^9 and Gln^4/Cys^6 systems of oxytocin and Leu^2/Gly^3 of NPLG. The chemical shifts are sufficiently close in oxytocin that full separation of the multiplets is not observed even at 360 MHz.

An optimum linear resolution enhancement filter (Ernst, 1966; Wittbold et al., 1980) was used to fix peak positions and thus facilitate convergence of line width and peak amplitude fitting routines. A time-domain weighing function $H(t) = [A \exp(-Lt)]/[(1 + q) \exp(1 - 2Lt)]$ was applied to the FID, where the parameter q determines the emphasis of the filter on resolution, $2L$ is the line width of the input Lorentzian, and A is a scaling factor. A value of 2.5×10^3 was used for q. In this application peak amplitudes after enhancement were not used, and the filtering routine was not amplitude preserving. Assignment of the filtered resonances was straightforward, given published values of $^3J(H'-H^\alpha)$ (Glickson, 1975) and the triplet structure of the Gly peptide NH resonance.

Once peak positions were available, line widths and peak amplitudes were obtained for the multiplets by a nonlinear least-squares simulation of the digitized frequency domain spectrum (Hamilton, 1964). For Gln^4/Cys^6 of oxytocin, the

Table I: Rate Parameters for Amide Hydrogen Exchange. Definitions[a]

parameter	symbol	defining relation
observed exchange rate	R	$-d[\text{amide-L}]/dt = R[\text{amide-L}]$
pseudo-first-order rate constants[b]		
acid-catalyzed exchange	k_L	$R = k_L[L^+] +$
base-catalyzed exchange	k_{OL}	$k_{OL}K_{L_2O}/[L^+]$
observed rate minimum[c]	R'	$R' = 2(k_L k_{OL} K_{L_2O})^{1/2}$
$[L^+]$ for which $R = R'$	L'	$L' = (k_{OL}K_{L_2O}/k_L)^{1/2}$

[a] L = H, D, or T. [b] A rate constant for general catalysis, k_0, is occasionally included. It is usually negligible. [c] At a given temperature.

downfield components of the doublets were not resolved by resolution enhancement ($\Delta\delta < 1$ Hz); this complete peak was therefore not used in the iteration, and kinetic analysis was based on the evolution of the two well-separated upfield components. The standard error of the calculated R's for these residues is slightly higher than for the others.

Analysis of Rates (Table I). The pseudo-first-order rate constants for exchange, k_D and k_{OD}, were obtained from the R's by a least-squares iteration based on eq 1. The weight

$$\ln R = \ln (k_D[D^+] + k_{OD}K_{D_2O}/[D^+]) \quad (1)$$

W assigned to each point was given by $W^{-1} = \delta \ln R = \delta R/R$, where δR is the standard error of R. The appropriateness of these weights derives from the preponderance of measurements near the pD′ and the exponential dependence of R on pD. The best-fit k's were used in the calculation of the pD–rate profile for each proton. Whereas R' and pD′ are obvious from examination of such a curve, k_D and k_{OD} are not; from the definitions of R, R', and [D′], one obtains (Hvidt & Corett, 1970)

$$\log R = \log R' + \log \left[\frac{1}{2}\left(\frac{[D^+]}{[D']} + \frac{[D']}{[D^+]} \right) \right] \quad (2)$$

demonstrating that R' and pD′ completely specify a profile and can be used in lieu of k_D and k_{OD} for comparative purposes. Equation 2 described a hyperbola symmetric about the line pD = pD′ with asymptotes of slope ±1, which may be positioned with its minimum at any point (−log [D″], log R'') in the pD–log R plane without alteration in shape. Departure from eq 1 and 2 is expected when the rate constants depend on pD, as the result either of titration of nearby functional groups or a pD-dependent conformational change.

An R factor ratio (Hamilton, 1964) was computed by allowing k_D and k_{OD} to vary jointly away from their fitted values to perform the error analysis for the k's. This amounts to vertical translation of the pD–rate profile in the plane. The error belts displayed with the profiles have the following significance: The composite hypothesis $k_D = k_D$, $k_{OD} = k_{OD}$ is rejected at the 0.05 confidence level if the profile calculated for k_D and k_{OD} falls above or below the belt. This assumes that variance in the k's is the consequence of pD-independent errors in the observed exchange rates, R.

The fitted rate constants were contrasted with values extrapolated for a random-coil peptide of identical amino acid sequence according to the nearest-neighbor model (Molday et al., 1972) (eq 3a and 3b), for the side chains pendant to

$$k_D(\text{pred}) = C_D(\rho)C_D(\lambda)k_D \text{ (PDLA)} \quad (3a)$$

$$k_{OD}(\text{pred}) = C_{OD}(\rho)C_{OD}(\lambda)k_{OD} \text{ (PDLA)} \quad (3b)$$

the residues immediately to the left and right, respectively,

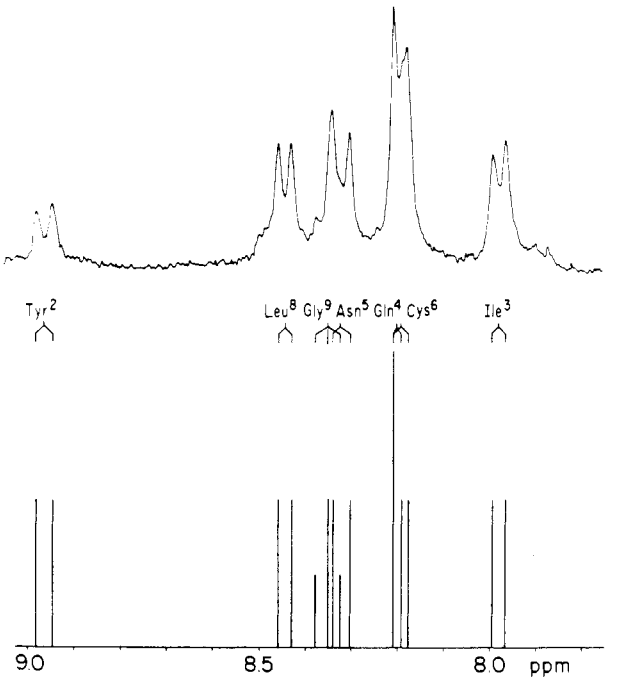

FIGURE 2: (Upper) The amide region of the 220-MHz ^{1}H NMR spectrum of oxytocin (8% w/v) at 19.5 °C, pD 1.90. (Lower) Diagrammatic representation of the resonance positions.

of a particular NH group (the amino acid sequence is arrayed in the usual sense).

When temperature and/or isotope corrections were required for comparison with kinetic data obtained elsewhere, eq 4a and 4b were employed. The ion-product term in eq 4b is

$$k_D(T) = 2.5k_H(T_0) \exp[\Delta G^{\ddagger}_H(T_0^{-1} - T^{-1})/R] \quad (4a)$$

$$k_{OD}(T) = 2.9k_{OH}(T_0)\frac{K_{H_2O}(T_0)}{K_{H_2O}(T)} \exp[\Delta G^{\ddagger}_{OH}(T_0^{-1} - T^{-1})/R] \quad (4b)$$

necessary because $\Delta G^{\ddagger}_{OH}$ was determined at constant pH (Englander & Poulsen, 1969). The numerical factors comprise the isotope correction and are chosen so that $R'(H_2O) \approx R'(D_2O)$, while pD′ ≈ pH′ + 0.4 at a given temperature (Molday et al., 1972; Molday & Kallen, 1972; Klotz & Frank, 1965).

Results

The chemical structures of oxytocin and NPLG are displayed in Figure 1. N-Terminal acetylation of the parent tripeptide was performed in order that the effect of the Pro^1 ND_2^+ on the exchange kinetics of Leu^2 NH be suppressed. The resonance overlaps in the amide region of the 220-MHz ^{1}H NMR spectrum of oxytocin were largely removed by resolution enhancement (Figure 2). Neither the isotopic constitution of the solvent nor the deuterium ion concentration between pD 1.9 and 5.5 significantly affected NMR chemical shifts and coupling constants, in agreement with previous measurements (Brewster & Hruby, 1973; Deslauriers et al., 1974b). The influence of these perturbations on the conformation of the hormone was therefore judged to be minimal.

The exchange parameters determined for PDLA by integration of the amide resonance agree well with previous measurements (Table II). Comparable spectra for representative oxytocin exchange runs are exhibited in Figure 3, and Figure 4 shows the result of simulation of the complex multiplets in the oxytocin spectrum at pD 3.04 by using the fitted R's. For six of the peptide protons of oxytocin (Ile^3 through Gly^9) and the two of NPLG, the variation of R with

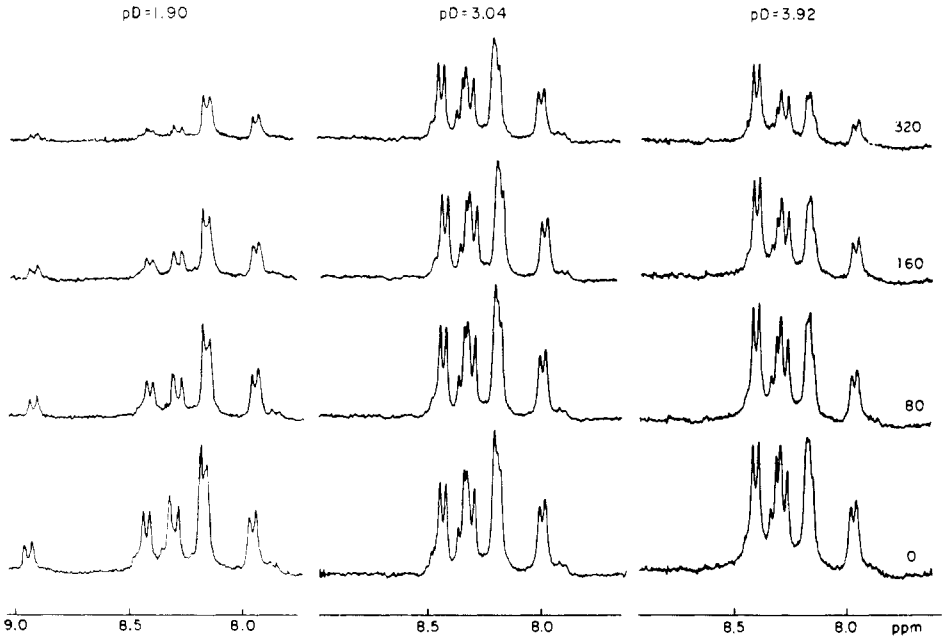

FIGURE 3: HDX of oxytocin. The temperature for all runs was 19.5 °C. The time in seconds is indicated above each row; time origin is arbitrary. The variation in pD′ among the residues is clearly visible.

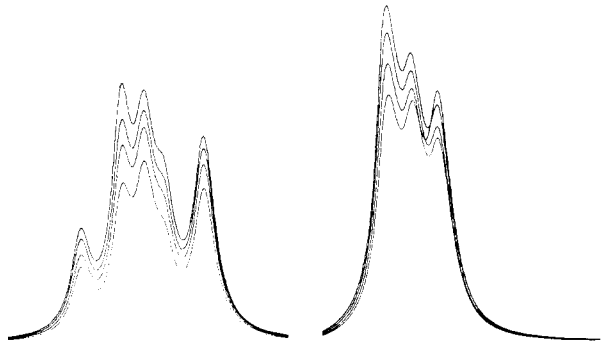

FIGURE 4: Simulation of the Asn[5]/Gly[9] (left) and Gln[4]/Cys[6] (right) multiplets at pD 3.04, with fitted values of R (see Materials and Methods).

Table II: Exchange Parameters for Poly(DL-alanine)[a]

technique	k_D	$10^{-10} \times k_{OD}$	pD′	R'
HDX, NMR	75.3	1.40	3.38	0.0628 (0.0555–0.0703)[b]
THX[c]	55.3	1.43	3.31	0.0543
HDX, IR[d]	61.6	1.44	3.33	0.0576

[a] k's are expressed in L mol^{-1} min^{-1} and R''s in min^{-1}. [b] The range for R' represents a 95% confidence interval (see Materials and Methods). The uncertainty in the pD′ is approximately ±0.1 unit. [c] k's given (Englander & Poulsen, 1969) were adjusted to 19.5 °C (D_2O) from 0 °C (H_2O) according to eq 3a,b, employing values for ΔG^*_H and ΔG^*_{OH} determined therein (15 and 17 kcal mol^{-1}, respectively). [d] Fitted to the observed values of R (Bryan & Nielsen, 1960) by eq 1 and adjusted to 19.5 °C from 22 °C by eq 3a,b without isotope factors. The same ΔG^*'s were used.

pD was adequately described by two pseudo-first-order rate constants, which are contrasted in Table III with values extrapolated from the kinetic parameters for PDLA (eq 3a and 3b). The experimental and predicted pD–rate profiles determined by the two sets of k's are displayed in sequence in Figures 5 and 6.

Tyr[2]. At pD 1.90, $R = 0.26 \pm 0.01$ min^{-1}. Exchange was too rapid to permit direct measurement at higher pD. This is consistent with the predominance of base-catalyzed HDX in all exchange runs. Displacement to lower pD of the pD–rate profile is characteristic of juxta-N-terminal residues (Scheinblatt, 1966). The single determination of R corresponds to a k_{OD} 4 times larger than the predicted random-coil value and close to the diffusion-limited rate (Eigen, 1964). Fivefold acceleration of this rate relative to prediction was reported from saturation transfer measurements (Krishna et al., 1979).

Table III: Exchange Parameters for Oxytocin and NPLG[a]

residue	observed[b] k_D	observed[b] $10^{-10} \times k_{OD}$	predicted[c] k_D	predicted[c] $10^{-10} \times k_{OD}$
Tyr[2]		4.4 × 10[2]		1.07 × 10[2]
Ile[3]	18.5	3.05	37.7	1.76
Gln[4]	25.9	2.03	47.5	1.98
Asn[5]	24.9	1.96	47.5	4.97
Cys[6]	11.7	4.23	26.7	12.5
Leu[8]	28.7	1.14	75.3	1.40
Leu[2] (NPLG)	28.2	1.01	75.3	1.40
Gly[9]	59.7	3.09	75.3	3.52
Gly[3] (NPLG)	57.8	2.64	75.3	3.52

[a] At 19.5 °C. k's are expressed in L mol^{-1} min^{-1}. [b] These are best-fit values. Confidence intervals for R' and pD′ obtained by joint variation of k_D and k_{OD} (see Materials and Methods) are displayed with the pD–rate profiles. [c] Extrapolated from our PDLA data and the appropriate nearest-neighbor corrections (Molday et al., 1972). The cysteine and Asn λ peptide corrections were used for Cys[6] and Gly[9], respectively.

Ile[3] *and Gln*[4] (*Figure 5a,b*). The experimental and predicted profiles correspond closely.

Asn[5] *and Cys*[6] (*Figure 5c,d*). The experimental profiles manifest moderate slowing with virtually no departure from the expected pD′. $\Delta G^{\ddagger}_D$ and $\Delta G^{\ddagger}_{OD}$ are each increased by $\Delta\Delta G^{\ddagger} = -RT \ln [R'(\text{obsd})/R'(\text{pred})]$, or 0.5 kcal mol^{-1} for Asn[5] and 0.6 kcal mol^{-1} for Cys[6].

Leu[8] (*Figures 5e and 6a*). While the experimental profile shows slight slowing relative to the PDLA-based extrapolation, it coincides with the profile for Leu[2] of NPLG.

Gly[9] (*Figures 5f and 6b*). It was necessary to approximate the λ corrections to the PDLA rate constants by the λ coefficients for asparaginyl (Molday et al., 1972). The experimental profile agrees closely with the projection thus obtained and coincides with that of the corresponding residue in NPLG.

The carboxamide protons of Gln[4], Asn[5], and Gly[9] were not detected in any exchange run. The R' and pD′ for terminal

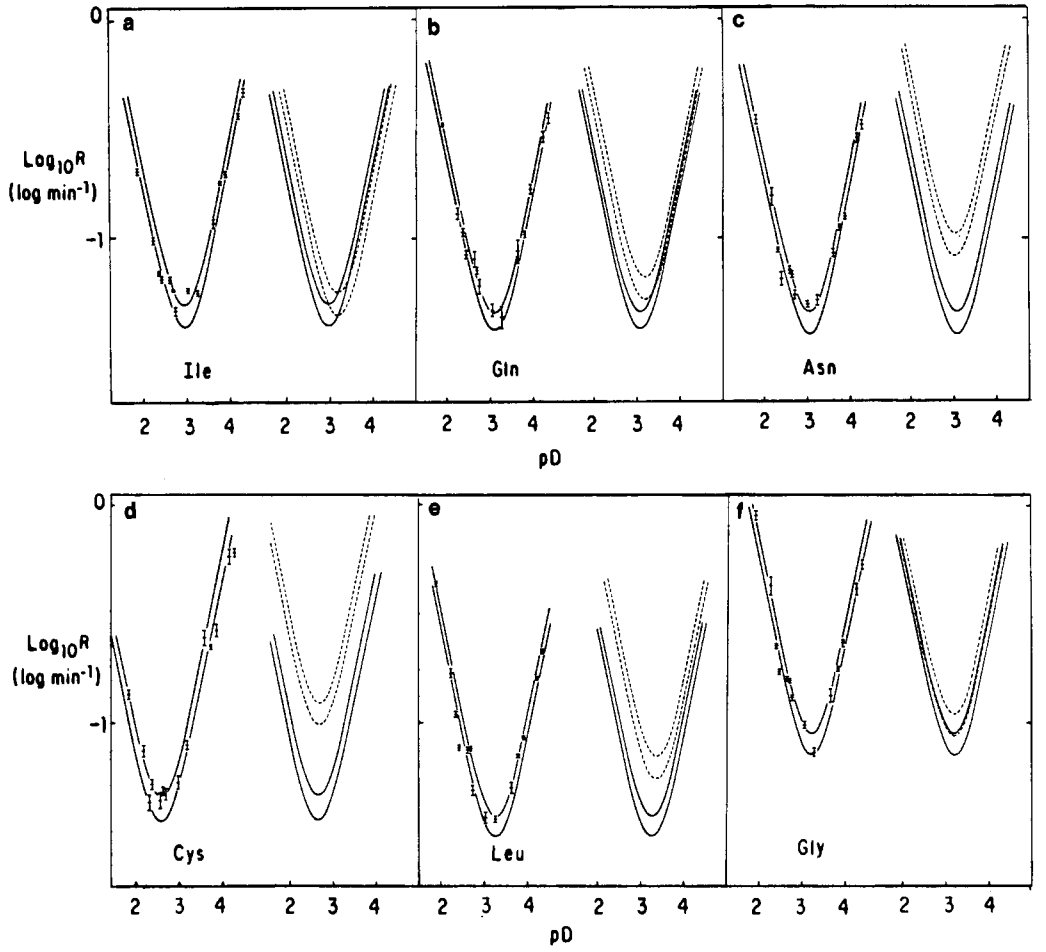

FIGURE 5: Exchange profiles for the peptidyl protons of oxytocin at 19.5 °C. The solid curves are calculated from the experimental rate constants; the dashed curves are calculated from rate constants extrapolated from the PDLA parameters (Table II) according to the nearest-neighbor model (Molday et al., 1972). The statistical significance of the belt representation is outlined under Materials and Methods.

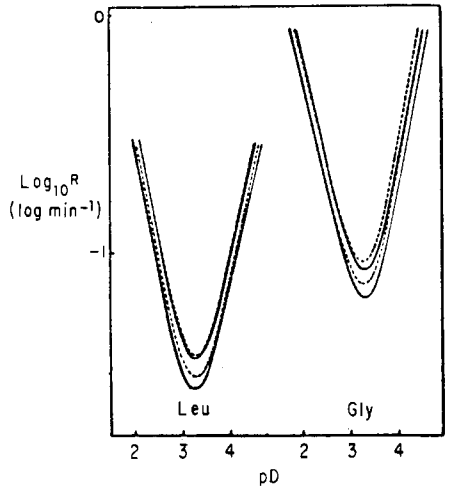

FIGURE 6: Exchange profiles for the peptidyl protons of NPLG (solid curves) at 19.5 °C. The dashed curves represent the equivalent residues in oxytocin.

carboxamides undergoing unhindered HDX at 19.5 °C should be approximately 1 min^{-1} and 4.5, respectively (Krishna et al., 1979; Molday et al., 1972); it is therefore concluded that exchange out was virtually complete prior to the start of data acquisition.

Discussion

The dependence of the exchange rates on pD for residues 3–9 in oxytocin show no significant departure from the rate law defining the pseudo-first-order rate constants, k_D and k_{OD} (Table I). It is therefore concluded that the structural influences on rates are independent of pD for these residues. The effects of neighboring substituent groups appear to be sufficient to account for the rate constants measured for the majority of the peptidyl protons. For the remainder, significant differences from the predicted pD–rate profiles are evident, which will be examined in detail.

The experimentally determined exchange parameters for Ile[3], Gln[4], Leu[8], and Gly[9] peptide NH conform closely to those projected for N-acetylated tripeptides of appropriate primary structure (eq 3a and 3b). The equivalence of exchange rates between NPLG and oxytocin corroborates (Deslauriers et al., 1974a; Live et al., 1979) further that the tocin ring and tripeptide tail are unassociated save at the point of juncture. These findings are compatible with appreciable structural flexibility in aqueous solution (Brewster & Hruby, 1973; Deslauriers et al., 1974a; Glickson, 1975; Kotelchuk et al., 1972; Glasel et al., 1973).

The discovery that base-catalyzed exchange for Tyr[2] NH is accelerated fourfold relative to the random-coil prediction is unanticipated and difficult to rationalize in terms of the known effects of peptide conformation on exchange rates. It has been asserted that structural factors act only to decrease k_{OL}, either by blocking access to the exchangeable proton or by lowering the ion product of water in its vicinity (Leichtling & Klotz, 1966); conformational influences which could cause this rate constant to exceed the random-coil value have not been described. Anomalous through-bond electronic effects exerted directly by nearby functional groups are unlikely to

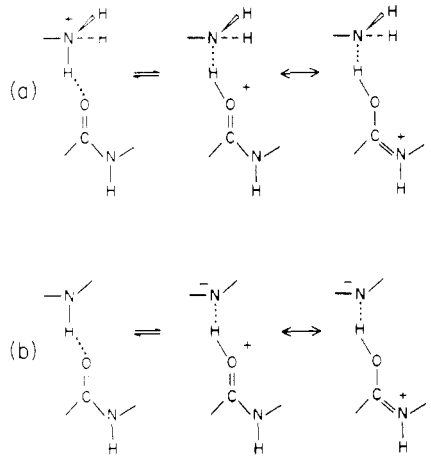

FIGURE 7: Stabilization of imidic acid tautomer of the peptide amide by partial proton transfer to carbonyl oxygen in a hydrogen-bonded complex. The proton on the N atom is as a result more susceptible to abstraction by base. (a) Donor moiety is an amide NH. (b) Donor moiety is an N-terminal amino group.

produce a discrepancy of this magnitude without measurably affecting the exchange profiles of neighboring residues, however, and thus cannot satisfactorily account for the observation. It has been suggested (Krishna et al., 1979) that the N-terminal amino group lies in close proximity to the Tyr^2 NH proton, accelerating the rate, but it is difficult to see how this should not *decrease* k_{OL} through competition between RNL_3^+ and the amide for available base, OL^-.

Enhanced acidity of the Tyr^2 NH proton should afford greater stabilization of the transition state for base-catalyzed exchange and result in a larger observed k_{OL} (Scheinblatt, 1970). This has been demonstrated in the case of inductive effects exerted by nearby substituent groups on amide exchange rates through study of a series of *N*-methylacetamide analogues (Molday & Kallen, 1972). Of the means by which electron density on the Tyr^2 N atom might be reduced purely by effects of the tertiary structure of the peptide, interaction of a proton donor with the N atom directly, while conceivable, appears unlikely because of its low basicity in neutral amides (Molday & Kallen, 1972). A more plausible mechanism involves the participation of the Cys^1 carbonyl oxygen in an intramolecular hydrogen bond as the acceptor moiety. The effect on k_{OL} is then mediated by the partial transfer of positive charge from the donor group to the O atom, stabilizing the imidic acid tautomer of the peptide function (Figure 7) and lowering the pK_a of the Tyr^2 NH (Llinas & Klein, 1975). Consistent with this hypothesis are the anomalously large chemical shift of the Tyr^2 amide proton in water, which could result from deshielding consequent to a shift in electron density away from the N atom in the hydrogen-bonded complex (Saito & Nukada, 1971), and the observation that Tyr^2 contains the sole peptidyl ^{15}N resonance in oxytocin found to be shifted downfield of the corresponding resonance in the N-acetylated free amino acid (Live et al., 1979). The same forces producing enhancement of base catalysis should diminish k_D to a comparable degree, resulting in a displacement of the exchange profile by 0.5–1.0 unit to lower pD, with lesser effect on R'. Quantitatively similar effects are produced by electron-withdrawing substituents (Molday et al., 1972; Berger et al., 1959). The donor group producing these kinetic effects cannot, a priori, be specified. Since the standard rates to which these kinetic data are compared were also measured in water, it seems evident that effects other than hydrogen bonding to water alone must be invoked and the involvment of other possible donors considered.

The contribution of similar proton-transfer complexes to hydrogen bonds in solution has been estimated from infrared spectroscopy (Vinogradov & Linnell, 1971) and by ^{14}N NMR (Saito et al., 1965). The occurrence of tautomeric shifts in connection with hydrogen bonding has been supported by ^{14}N NMR chemical shift correlations and increased rotational barriers about the C–N bond axis (Saito et al., 1971) and by solvation effects on the ^1H, ^{13}C, and ^{15}N NMR chemical shifts of amide groups in rigid peptides (Llinas & Klein, 1975; Llinas et al., 1976, 1977). The exact effect of intramolecular hydrogen bonding on exchange kinetics at the acceptor site does not appear to have been experimentally investigated. At present, no other heteropolypeptide for which residue-specific exchange profiles have been obtained is known to possess stable intramolecular hydrogen bonds in aqueous solution, although the data for alumichrome is suggestive (Llinas et al., 1973) and that for gramicidin S in water–dioxane (Philson & Bothner-By, 1980) may be relevant. For homopolymers, helical poly(L-glutamic acid) is relatively insoluble in water at acid pH (Klotz, 1968), and only limited data on its exchange kinetics in aqueous media are available (Welch & Fasman, 1974). In dioxane–D_2O (1:1), complete exchange profiles have been derived (Leichtling & Klotz, 1966). By use of measured rate constants for *N*-methylacetamide (Klotz & Frank, 1965) as a basis for the extrapolation, the experimental pD′ (3.2) corresponds to a pD′ of 2.45 in pure D_2O. In contrast a pD′ of 3.0 is predicted for poly(L-glutamic acid) by correcting the PDLA exchange parameters for the inductive effect of the unionized glutamyl side chains (Molday et al., 1972). This apparent relative enhancement of base catalysis compared to acid catalysis is consistent with a model in which the acidity of each exchanging proton in increased by hydrogen bonding to the adjacent carbonyl oxygen. Further study of model compounds in water is necessary to establish whether amide–amide hydrogen bonding indeed produces these predicted displacements of exchange profile.

Unlike the result for Tyr^2, the two- to threefold slowing of exchange observed for the peptidyl protons of Asn^5 and Cys^6 appears to provide a more conventional example of the effects of peptide folding on exchange rates; for both residues, the pD′ is well approximated by the random-coil prediction. Previous attempts to provide a structural rationale for anomalously low amide hydrogen exchange rates in peptides (Llinas et al., 1973; Laiken et al., 1969; Galardy et al., 1971; Bleich et al., 1973) have utilized the formalism (Linderstrøm-Lang, 1955) according to which the conformation of a molecular exchange site is either "open" (I state; unhindered exchange) or "closed" (N state; no exchange), and the ratio k_{obsd}/k_{pred} depends only on the thermodynamics of the $I \rightleftarrows N$ transition. The stipulation that no exchange occur from the N state may be misleading, however, especially in light of recent studies (Bernasconi & Carre, 1979; Crooks, 1975) demonstrating diminished proton transfer rates in certain small molecules which are unlikely to assume conformations in which transfer is abolished entirely, or, alternatively, in which the proton transfer site is always at least partially solvated. A more flexible model (eq 5) therefore retains the two-state formalism

$$UH^* \underset{BH}{\overset{k_u}{\leftarrow}} UH \underset{BH^*}{\overset{k_r}{\rightleftharpoons}} RH \rightarrow RH^* \qquad (5)$$

but permits the difference between the activation energies for exchange from the open and close conformations to vary. Here, exchange proceeds at the random-coil rate in the unrestricted (U) state, while in the restricted (R) state it is to some degree limited by local constaints imposed by tertiary

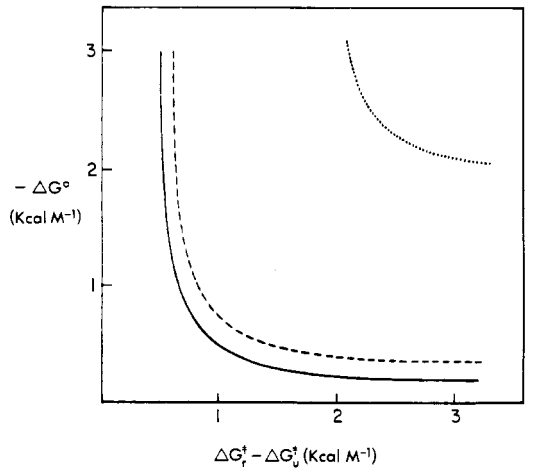

FIGURE 8: Predicted values of $-\Delta G^\circ = RT \ln K$ for the U $\rightleftarrows$ R transition: two-level model calculated from eq 8 at T = 19.5 °C. (Solid line) Asn^5, $\Delta\Delta G^\ddagger$ = 0.5 kcal/mol^{-1}. (Dashed line) Cys^6, $\Delta\Delta G^\ddagger$ = 0.6 kcal mol^{-1}. (Dotted line) Hypothetical, $\Delta\Delta G^\ddagger$ = 2.0 kcal mol^{-1}. The Linderstrøm-Lang approximation is adequate for Asn^5 and Cys^6 when $\Delta G^\ddagger_r - \Delta G^\ddagger_u > 2$ kcal mol^{-1}.

structure. If conformational equilibrium is achieved relatively rapidly compared to the rate of exchange, the observed exchange rate is given by (Curtin, 1954)

$$k_{obsd} = \frac{k_u + k_r K}{1 + K} \tag{6}$$

where $K \equiv [RH]/[UH]$. In the special case $k_r \ll k_u$, eq 6 simplifies to $k_{obsd} = k_u/(1 + K)$, or

$$K = k_u/k_{obsd} - 1 \approx R'_{pred}/R'_{obsd} - 1 \tag{7}$$

This is equivalent to the Linderstrøm-Lang, expression, EX_2 case (Hvidt & Nielsen, 1966). In general, $k_u/k_r = \exp(\Delta G^\ddagger_r - \Delta G^\ddagger_u)/RT$, where the activation energy difference depends on the nature of the R state. Values of several kilocalories per mole or more are expected for $\Delta G^\ddagger_r - \Delta G^\ddagger_u$ when exchange is blocked by intramolecular hydrogen bonding (Eigen et al., 1962) or when the NH proton is so well isolated as to render it unavailable for bridging hydrogen bonds to the solvent, without necessarily serving as an intramolecular hydrogen bond donor. Values of 1 kcal mol^{-1} or less, on the other hand, are consistent with an R conformer in which solvent approach to the exchange site is partially blocked. It is possible, by rearrangement of eq 6, to relate the apparent exchange barrier, $\Delta\Delta G^\ddagger \equiv \Delta G^\ddagger_{obsd} - \Delta G^\ddagger_u \approx -RT \ln R'_{obsd}/R'_{pred}$, to $\Delta G^\ddagger_r - \Delta G^\ddagger_u$ and K directly:

$$\Delta\Delta G^\ddagger = -RT \ln \left[\frac{1 + Ke^{-(\Delta G^\ddagger_r - \Delta G^\ddagger_u)/RT}}{1 + K} \right] \tag{8}$$

For the peptidyl protons of Asn^5 and Cys^6 of oxytocin, values of 0.45 and 0.39, respectively, were obtained for R'_{obsd}/R'_{pred}. By use of the Linderstrøm-Lang equation, modest values of 1.2 and 1.6 are calculated for K. However, because there is no reason to assert a priori that $\Delta G^\ddagger_r - \Delta G^\ddagger_u$ is large, eq 8 should be employed and K related to $\Delta G^\ddagger_r - \Delta G^\ddagger_u$ as the latter varies for any choice of $\Delta\Delta G^\ddagger$. This is done in Figure 8; clearly as $\Delta G^\ddagger_r - \Delta G^\ddagger_u$ approaches $\Delta\Delta G^\ddagger$, K increases without limit, and the same kinetic result for Asn^5 and Cys^6 can be viewed as consistent with prevalent local conformations in which solvent access is sterically blocked to a moderate degree.

Several methods may be applied to the problem of discriminating between hydrogen bonding and steric blocking as the source of shielding from solvent. The effects of temperature on exchange kinetics have been investigated (Llinas et al., 1973) since different enthalpic and entropic contributions to the energy of activation for exchange might be expected. In aqueous solution, it is very difficult to make sufficiently precise measurements because of the restricted temperature range, and the thermodynamic interpretation may not be definitive (Hvidt & Nielsen, 1966). Anomalous NMR chemical shifts and chemical shift temperature coefficients have been considered, but for proton resonances similar objections to those cited above have been raised. Nitrogen chemical shifts, on the other hand, appear to be well correlated with the degree of C–N double bond character which in turn strongly depends, in amides, on the degree of hydrogen bonding to the acceptor carbonyl and from the donor NH (Levy & Lichter, 1979; Saito et al., 1971). In oxytocin, the peptidyl ^{15}N resonance for Asn^5 in water is displaced 6.3 ppm upfield of the corresponding resonance in the N-acetylated free amino acid in Me_2SO (Live et al., 1979), and this has been interpreted as a net deficiency of hydrogen bonding at this site (via intramolecular or solvent interactions). This strongly suggests that the observed slowing for Asn^5 results from local steric restriction and not from intramolecular hydrogen bonding. No such distinction is available for Cys^6, which shows a ^{15}N shift close to the expected value.

A third set of methods might be based on specific identification of the acceptor moiety for the hydrogen bond. The suggestion has been made above that displacement of an exchange profile to acid pH is expected for an amide proton adjacent to a carbonyl group serving as an hydrogen bond acceptor. This effect is, in theory, capable of identifying such an acceptor site by kinetic criteria. If the kinetic model formulated in eq 5 and 6 is applied to both donor and acceptor sites, the equilibrium constants for the U $\rightleftarrows$ R transition calculated for the two sites may be directly compared. In oxytocin, such a comparison may be made to determine whether the presently unexplained slowing at Cys^6 and the acceleration at Tyr^2 are to be regarded as manifestations of a single transannular hydrogen bond between Cys^6 NH and Cys^1 CO, with a single formation constant K.

Recasting eq 6 for the two sites, one obtains

$$K_{acc} = (k_{u,acc} - k_{obsd,acc})/(k_{obsd,acc} - k_{r,acc}) \tag{9a}$$

$$K_{don} = (k_{u,don} - k_{obsd,don})/(k_{obsd,don} - k_{r,don}) \tag{9b}$$

where K_{acc} and K_{don} are the equlibrium constants for formation of the R state, here the hydrogen-bonded complex, computed for the acceptor and donor moieties, respectively. The acceptor site rate constants refer to the adjacent amide proton, Tyr^2; for base-catalyzed exchange, $k_{r,acc} > k_{u,acc}$. The value of 1.6 for K_{don} obtained from the Linderstrøm-Lang expression clearly applies in the present case since $k_{r,don} \ll k_{obsd,don} < k_{u,don}$. K_{acc}, on the other hand, depends on an accurate assessment of $k_{r,acc}$, because $k_{obsd,acc} \lesssim k_{r,acc}$. An obvious upper bond for the latter is the diffusion-limited rate, which is $\sim 5 \times 10^{12}$ M^{-1} min^{-1} for the recombination of D^+ and OD^- (Eigen et al., 1962). According to the Eigen formulation of proton-exchange rates (Eigen, 1964), the pK_a of Tyr^2 NH must be lowered by 2 units to bring the primary structure corrected k_{OD} for this residue to within 5% of the diffusion-controlled limit, a shift consistent with those induced by electron-withdrawing substituent groups (Molday et al., 1972; Molday & Kallen, 1972) and with the pK_a's of N-substituted imidic acid esters (Hartigan & Cloke, 1945; Pletcher et al., 1968). By use of this estimate for $k_{r,acc}$, eq 9a then yields K_{acc} = 2.3. Thus, if the equilibrium constants for hydrogen bond formation calculated

for the donor and acceptor sites are directly comparable, the determination that $K_{acc}/K_{don} \simeq 1$ indicates that an essentially thermoneutral transannular hydrogen bond between Cys^6 NH and Cys^1 CO is indeed consistent with the kinetic data. Such a bond was recently proposed, among others, for oxytocin in aqueous solution, albeit without experimental evidence (Krishna et al., 1979). It should be emphasized that until means are available for the selective observation of intramolecular hydrogen bonds in macromolecules, it is not possible to exclude with certainty the likelihood of participation of Cys^6 NH in other weak transannular hydrogen bonds without appreciable proton transfer character or in simple site sequestration.

Of alternative donor moieties which might be considered for the putative hydrogen bond to Cys^1 CO in oxytocin, the protonated N-terminal amino group is a surprisingly good candidate. It is a much stronger proton donor than secondary amides, favoring proton-transfer complex formation (Vinogradov & Linnell, 1971). The anomalous long-range pH-induced ^{13}C NMR shifts that have been observed as the N-terminal amino group is titrated through its pK_a (Deslauriers et al., 1974b) appear to reflect the importance of its state of ionization to the overall conformation of the hormone or the cyclic moiety. Finally, the bulky C$^\alpha$ substituent on Cys^1, with the proximate disulfide bridge, should result in relative stabilization of the cis and gauche orientation of the N-terminal amino group and Cys^1 carbonyl O atom; such conformers are conducive to intramolecular hydrogen bonding, perhaps involving a bridging water molecule. Similar orientational preferences have been indicated for tripeptide with bulky N-terminal residues by downfield displacement of the central ^{15}N resonance when the terminal amino group is deblocked (Markowski et al., 1977; Saito et al., 1971).

In summary, it has been shown that resolution enhancement filtering and suitable interactive methods greatly facilitate the selective measurement of rates in kinetic NMR experiments. The results affirm that in aqueous solution extensive regions of the peptide backbone of oxytocin are completely solvated, as indicated by the random-coil exchange rates of the peptidyl protons of Ile^3, Gln^4, Leu^8, and Gly^9. These regions are believed on the basis of measured NMR coupling constants (Wyssbrod et al., 1977; Glickson et al., 1976; Brewster & Hruby, 1973) and relaxation times (Deslauriers et al., 1974b) to be relatively flexible. On the other hand, the tertiary structure of the peptide exerts a measurable influence on exchange kinetics at Tyr^2, Asn^5, and Cys^6. It is argued that the observed slowing for Asn^5 NH is most likely the result of steric hindrance to solvent access. The anomalously high k_{OD} for Tyr^2 NH appears best explained by partial proton transfer to the Cys^1 carbonyl O atom, and the possibility of identifying acceptor moieties of intramolecular hydrogen bonds in peptides by the detection of acid-displaced hydrogen-exchange profiles is thereby introduced. The Cys^1 C=O may in fact be the acceptor site in a thermoneutral hydrogen bond to Cys^6 NH; evidence of this specific interaction is inferential, however, and the protonated N-terminal amino group is also considered a likely donor. It should be emphasized that the conformational forces influencing the hdyrogen-exchange kinetics of Asn^5 and Cys^6 NH are weak and unlikely to contribute appreciably in the stabilization of the prevailing conformers of oxytocin at equilibrium in aqueous solution.

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Structural Changes in Synthetic Myosin Minifilaments and Their Dissociation by Adenosine Triphosphate and Pyrophosphate†

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ABSTRACT: Morphologically similar short myosin and rod filaments (minifilaments) have been prepared in 10 mM Tris–citrate buffer, pH 8.0, in the absence of other myosin or rod forms. Both minifilament systems are dissociated in the same manner in the presence of ATP or pyrophosphate. Identical binding of these ligands to myosin and rod minifilaments suggests that myosin heads play no role in substrate-induced destabilization of the minifilaments. The effects of ATP and pyrophosphate on minifilaments are similar to their dissociating effect on synthetic filaments [Harrington, W. F., & Himmelfarb, S. (1972) *Biochemistry 11*, 2945–2952], thus justifying their use in conformational studies in lieu of filaments. In view of their small size and homogeneity, the minifilaments constitute an appropriate material for such studies. The binding of pyrophosphate to myosin and rod minifilaments decreases their α-helical content, as measured by circular dichroism. No change in the secondary structure of subfragment 1 and light meromyosin is observed upon binding of pyrophosphate, but substantial changes (10%) are detected in subfragment 2. The structural changes in myosin, possibly relevant to contraction, are localized in the subfragment 2 region of the molecule. These results emphasize the importance of charge interactions in the functional behavior of thick filaments.

Electrostatic interactions within the myosin filament have been considered by many authors to be an important element of contractile events. In a contraction model developed by Morales & Boots (1953) and Morales (1955), the negative charge of adsorbed ATP molecules would lead to an extension of a segment of the myosin heavy chain. Following cleavage of the terminal phosphate, the local charge would be reduced, resulting in contraction of the chain and consequent force generation. The search for corresponding conformational changes in myosin induced by ATP binding and cleavage or by interaction with the competitive inhibitor, pyrophosphate, produced no evidence in support of any transitions except those

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