# Amide Proton Exchange Rates in a Cyclic Dodecapeptide of Defined Conformation. pH and Conformation Dependence†

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ABSTRACT: Peptide proton and side chain carboxamide proton exchange rates of the cyclic dodecapeptide *cyclo*-(Met-Val-Gly-Pro-Asn-Gly)$_2$ were determined over the pH range 1.2–9 by NMR methods. The rate constants of the base-catalyzed exchange reactions range from 2.5 to 0.006 times those predicted from measurements on *N*-acetyl *N'*-methylamides of the corresponding residues, and the factors for each residue are consistent with the proposed conformation of the cyclic peptide. The rate constants of the acid-catalyzed exchange of the backbone protons are not clearly dependent on conformation and are 10–20 times slower than those calculated from the model compounds. Since the side chain exchange rates in acid are similar to the corresponding rates in model substances, it is suggested that backbone conformational stability provides a barrier to the rehybridization at nitrogen necessary for exchange via N-protonation.

Proton exchange rates of peptide bonds have for some years been a valued tool for exploring protein conformation, since they are affected by solvent accessibility, as modulated by conformational flexibility, and by the stability of intramolecular hydrogen bonds (Hvidt & Nielsen, 1960; Englander et al., 1972; Englander, 1975; Woodward & Hilton, 1979). They have also been used, less extensively, to study conformational questions in smaller peptides. In contrast to proteins, the peptides that have been studied are not large enough to bury all aspects of a CONH unit, and most of them have considerable backbone flexibility. There are accurate kinetic data for small peptide model systems from which questions about the mechanism of exchange processes are answered for sterically unhindered systems (Molday et al., 1973), but there is not much information about conformation-induced steric influences on the elementary steps, since few oligopeptides of known conformation have been studied in a detailed way. Although cyclic peptides of sufficient complexity and conformational stability can be used to provide information about peptide bonds in a variety of defined steric situations, a review of the literature indicates that only for the cyclic decapeptide gramicidin S (Philson & Bothner-By, 1979; Krauss & Chan, 1982) are there measurements that provide good rate constants for the acid- and base-catalyzed exchange reaction of backbone protons. We considered that studies of an additional conformationally stable cyclic peptide of different backbone folding could materially add to understanding the mechanism of exchange and the influence of conformationally related steric effects on exchange.

We therefore made detailed kinetic studies of proton exchange in *cyclo*-(Met-Val-Gly-Pro-Asn-Gly)$_2$. NMR studies of this peptide suggest that it has a stable backbone conformation (Kopple & Go, 1977): The ranges of the NMR observables, such as coupling constants, chemical shifts, and evidence of solvent exposures, are large, indicating that conformational averaging is inhibited. The values themselves are consistent with the same backbone folding in water, methanol, dimethyl sulfoxide, and hexafluoro-2-propanol solutions, indicating that the dominant conformation is intrinsically stable.

The backbone proposed for *cyclo*-(Met-Val-Gly-Pro-Asn-Gly)$_2$ is shown in Figure 1. The N–H protons of Met-1 and both Gly-3 and Gly-6 residues are sequestered from the solvent. The dotted lines in Figure 1 indicate the likely proximity of hydrogen-bonding groups, although as usual there is no direct NMR evidence for particular hydrogen bonds. The side chain–backbone hydrogen bond shown at Asn is suggested by the dominant side chain rotamer found for that residue. A search through conformation space with calculation of conformational energies has indicated that the NMR-based conformation is a stable one (Madison, 1978). In the computed conformation, the good intrapeptide hydrogen bonds are those 1–1 across the Met unit, 1–3 from Met C=O to Gly-3 N–H, and from the Asn side chain C=O to the Asn backbone N–H. Gly-6 N–H is also sequestered.

We considered *cyclo*-(Met-Val-Gly-Pro-Asn-Gly)$_2$ a good peptide for study because it contains three kinds of CONH group placement relative to the cyclic backbone. Three CONH groups have internally directed N–H bonds and peripherally directed C=O, one has the reverse placement, and one has the CONH plane perpendicular to the average backbone plane. Effects of C=O and N–H exposure may thus be compared for acid- and base-catalyzed exchange. In addition, because the peptide contains no groups that ionize, its conformation will be independent of pH in the range necessary for the exchange study.

## Experimental Procedures

The synthesis, characterization, and spectral assignments for *cyclo*-(Met-Val-Gly-Pro-Asn-Gly)$_2$ have been reported (Kopple & Go, 1977). Before use in this work, the peptide was rechromatographed on a Bio-Gel P-4 column, using 2% acetic acid as the eluant; subsequent high-pressure liquid chromatography (HPLC) analysis ($\mu$Bondapak C-18 column, 20% acetonitrile, detection at 204 nm) showed only a single component.

The samples used for NMR studies were 3 mM in peptide, 20 mM in NaCl, and 5 mM in buffer. Bisulfate (pH 2.2–2.5), oxalate (pH 1.2–2.8), malonate (pH 3.5–7.4), and borate (pH 7.0–9) were titrated with NaOH or HCl solutions to the appropriate pH. Some experiments at pH 1.1–1.9 utilized only hydrochloric acid. The pH of each sample was measured in the NMR sample tube by using a calibrated combination electrode before and after each NMR run.

The solvent for all experiments was $H_2O$ containing 10% $D_2O$. Samples were not degassed. The sample tube size was 5 mm.

† From the Department of Chemistry, Illinois Institute of Technology, Chicago, Illinois 60616. *Received April 27, 1983.* This research was supported by the National Institute of General Medical Sciences (Grants GM 26071 and 26071-02S1). It is taken from the Ph.D. thesis of V.P.N.

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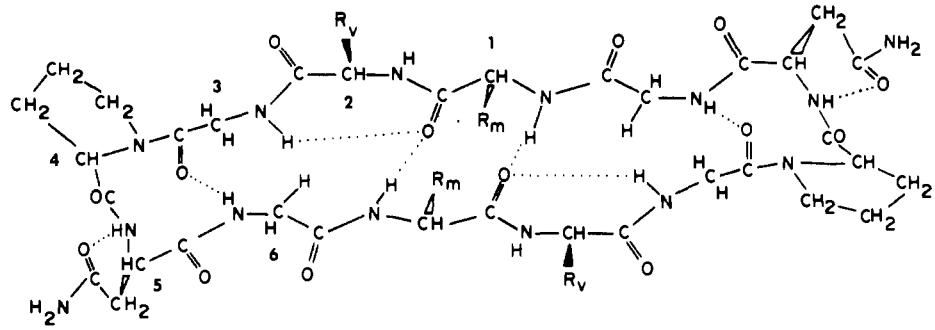

FIGURE 1: Likely conformation of *cyclo*-(Met-Val-Gly-Pro-Asn-Gly)$_2$. The residue numbering used in the text is shown.

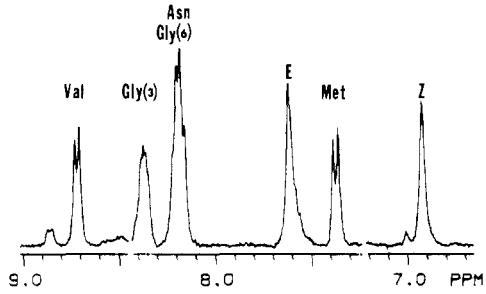


FIGURE 2: N–H proton region (base line straightened) of the 300-MHz proton spectrum of *cyclo*-(Met-Val-Pro-Gly-Asn-Gly)$_2$ in $H_2O$, 21 °C. The assignment of Gly-3 and Gly-6 is that made in Kopple & Go (1977).

NMR experiments were performed by using a Nicolet spectrometer operating at 300 MHz for protons. The temperature of the samples was maintained at 21 ± 1 °C by the standard temperature control system of the instrument. For pseudo-first-order rate constants of 2–120 $s^{-1}$, the rates were determined from line broadening. Between 0.2 and 15 $s^{-1}$, saturation transfer was used, and for rates between $10^{-4}$ and $10^{-3}$ $s^{-1}$, the growth of resonances was observed as deuterium was exchanged out. All spectra were observed by using the Redfield 2-1-4 soft pulse sequence (Redfield et al., 1975; Redfield, 1978). The carrier frequency was near the center of the amide proton resonances, 1000 Hz downfield from the water resonance. Figure 2 shows the spectrum of the N–H region.

For the line-broadening experiments, a reference line width was obtained from a sample adjusted to pH 5.0, where exchange does not contribute to line width. Higher pH samples of the same height were then inserted at the same position in the probe and their spectra measured. The exchange rates were determined by using

$$R = \pi[\nu_{1/2} - \nu_{1/2}(\text{ref})]$$

In these experiments, the half-height widths $\nu_{1/2}$, were measured across the full N–H doublet or triplet; 0.2-Hz line broadening was used.

The saturation transfer experiments consisted of two measurements, determination of $T_1$ and determination of saturation transfer. $T_1$ was measured by using the saturation–recovery technique. The amide protons were saturated by irradiation with unmodulated continuous wave (cw) decoupler power centered in the N–H resonance region of the spectrum. Sufficient power was used to saturate resonances over a spectral region 600 Hz wide in 1 s. Observation after the variable delay was by the 2-1-4 pulse sequence. The $T_1$ values were calculated by the Nicolet software using peak intensities. Spectra were obtained for 6–10 delay times after saturation. Except for the data from the overlapping Gly-6 + Asn N–H resonances (see Figure 2), all of the first-order relaxation plots were reasonably linear. In the latter case, the curved plot could be approximated as the sum of two exponential decays; however, this analysis was only performed at pH 6–7, where the two $T_1$'s obtained differed by a factor of ca.6. The $T_1$ values obtained are tabulated in Table I.

Table I: Spin–Lattice Relaxation Times of N–H Protons in *cyclo*-(Met-Val-Gly-Pro-Asn-Gly)$_2$ as a Function of pH at 21 °C

| | spin–lattice relaxation time (ms) | | | | | |
|---|---|---|---|---|---|---|
| pH | Val | Gly-3 | Met | $NH_2$(E) | $NH_2$(Z) | Gly-6/Asn |
| 1.1 | 228 | 260 | 265 | 57 | 52 | |
| 1.6 | 225 | 246 | 275 | 86 | 76 | |
| 1.6 | 200 | 237 | 271 | 73 | 66 | |
| 2.2 | 228 | 272 | 282 | 147 | 127 | |
| 2.2 | 227 | 251 | 286 | 150 | 132 | |
| 2.5 | 181 | 219 | 231 | 150 | 127 | |
| 4.6 | 210 | 263 | | 218 | 184 | |
| 6.6 | 174 | 242 | 248 | 156 | 164 | 286/50 |
| 7.0 | 141 | 237 | 223 | 118 | 141 | 270/48 |
| 7.4 | 96 | 252 | 188 | 75 | 115 | |
| 7.6 | 91 | 233 | 166 | 68 | 104 | |
| 7.7 | 73 | 237 | 166 | 65 | 111 | |
| 8.1 | 33 | 194 | 83 | 21 | 54 | |
| 8.5 | | 151 | 54 | | 52 | |
| 9.0 | | 69 | 36 | | 28 | |

Saturation transfer was measured under steady-state conditions: The ratio of resonance intensities with the decoupler cw power into the $H_2O$ resonance, about 1000 Hz above the center of the amide proton region, and an equal distance below the N–H region, 2000 Hz below the $H_2O$, was determined. The decoupler was on for 2.5 s and gated off during acquisition, and the 2-1-4 sequence was used for observation. Decoupler power was adjusted well below the level necessary to produce detectable saturation of the N–H resonances when the decoupler was on the low-field side. The intensity ratios so determined are given in Table II. The first-order rate constants were calculated from these ratios by using

$$k_{\text{obsd}} = \left(1 - \frac{1}{p}\frac{M}{M_0}\right)\frac{1}{T_1}$$

where $M/M_0$ is the ratio measured at a given pH and $p$ is the $M/M_0$ ratio observed at those pHs where exchange is negligibly slow relative to $T_1$. $p$ represents the solvent- (water) induced nuclear Overhauser enhancement (NOE). The NOE values can be obtained from Table II.

In this rate range, $k_{\text{obsd}}$ can also be estimated from

$$k_{\text{obsd}} = \frac{1}{T_1(\text{obsd})} - \frac{1}{T_1(\text{ref})}$$

where $T_1$(ref) is obtained from measurements at pHs at which exchange is too slow to affect the relaxation, i.e., in the regions in Table I where $T_1$ is pH independent. Values of $k_{\text{obsd}}$ so obtained were not regularly used in calculating second-order

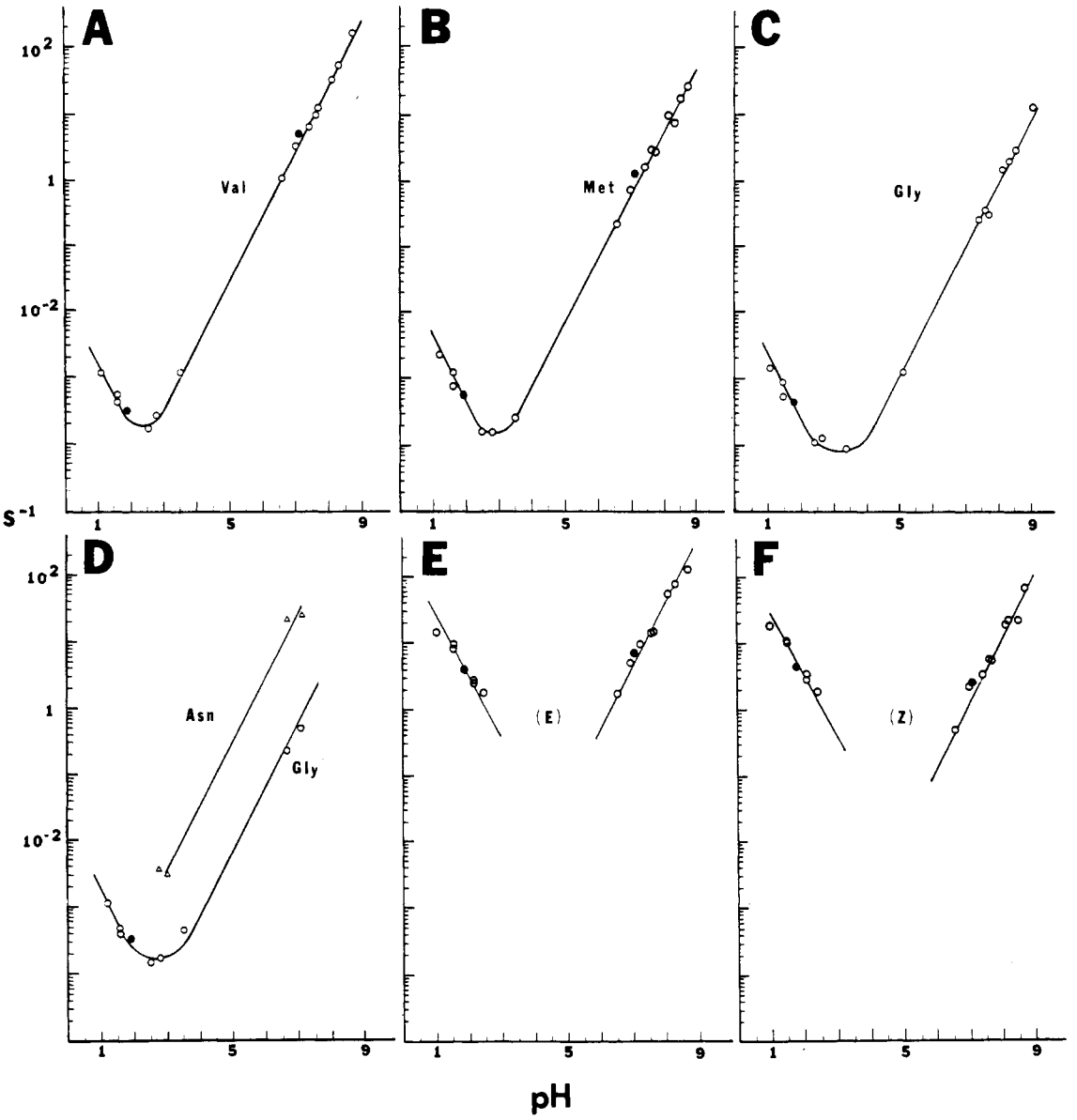


FIGURE 3: First-order exchange rate constants of amide and peptide protons in *cyclo*-(Met-Val-Gly-Pro-Asn-Gly)$_2$ at 21 °C as a function of pH. (A) Val; (B) Met; (C) Gly-3; (D) Asn (backbone) (triangles) and Gly-6 (circles); (E) Asn side chain, E proton; (F) Asn side chain, Z proton. Filled circles are at higher salt concentration.

rate constants for the base-catalyzed reaction, although they were quite close to those obtained from the saturation transfer equation. However, saturation transfer could not be measured for the overlapping Gly-6 and Asn N–H lines, but separate $T_1$ values could be estimated in some cases. Therefore, first-order rate constants for these residues at pH 6.6 and 7.0 were obtained from $T_1$'s by using $T_1$(ref) estimated as 280 ms.

Measurements of the slowest exchange rates were carried out as follows. Samples of peptide were made up in $D_2O$, stored long enough for complete exchange of acidic protons, and lyophilized in the NMR sample tubes. Immediately before insertion into the magnet gap, they were mixed with appropriate buffer solutions made up in 10% $D_2O$. The increase in intensity of the N–H resonances was monitored by using the 2-1-4 pulse sequence. The rate constant was determined from a plot of ln $(A_{inf} - A)$ vs. time. For the superimposed Gly-6 + Asn resonances, the data could be analyzed at some pHs as the sum of the two first-order processes differing by about a factor of 20 in the rate constant. It was established by difference spectra that the faster exchanging proton of the overlapping two was that of Asn and the slower that of the Gly-6.

The first-order exchange rate constants obtained by the above methods were plotted as a function of pH. These plots are shown in Figure 3. Second-order rate constants for acid and base catalysis, $k_H$ and $k_{OH}$, were obtained by fitting the points in the appropriate leg of the plots to the following equations:

$$\log k_H = \log k_{obsd} + \text{pH}$$

$$\log k_{OH} = \log k_{obsd} + \text{pOH}$$

log $K_{water}$ ($K_W$) was taken to be 14.13 at the temperature of the measurements, 21 °C (Bjerrum, 1929). The rate constant for the uncatalyzed reaction, $k_{aq}$, was obtained from the observed rate at the pH of its minimum:

$$\text{pH}_{min} = -\tfrac{1}{2} \log (k_{OH} K_W / k_H)$$

The contribution of added 2,2,6,6-tetramethylpiperidinyl-1-oxy to the $T_1$ relaxation of the N–H protons was measured in a malonate-buffered pH 4.6 sample 20 mM in NaCl and 6.1 mM in peptide by saturation recovery. Five nitroxyl concentrations between 0 and 9.2 mM were used. Second-order rate constants for relaxation were obtained from the

Table II: $M/M_0$ Values for the N-H Protons of *cyclo*-(Met-Val-Gly-Pro-Asn-Gly)$_2$ as a Function of pH at 21 °C[a]

| | $M/M_0$ | | | | |
|---|---|---|---|---|---|
| pH | Val | Gly-3 | Met | $NH_2$(E) | $NH_2$(Z) |
| 1.1 | 1.24 | 1.10 | 1.13 | 0.33 | 0.24 |
| 1.6 | 1.23 | | 1.11 | 0.40 | 0.40 |
| 1.6 | 1.21 | 1.11 | 1.12 | 0.40 | 0.41 |
| 1.6 | 1.21 | 1.11 | 1.10 | 0.41 | 0.39 |
| 2.2 | 1.25 | 1.15 | 1.18 | 0.72 | 0.73 |
| 2.2 | 1.23 | 1.19 | 1.08 | 0.74 | 0.70 |
| 2.2 | 1.23 | 1.19 | 1.13 | 0.72 | 0.76 |
| 2.3 | 1.21 | 1.19 | | 0.66 | 0.74 |
| 2.5 | 1.23 | 1.17 | 1.14 | 0.86 | 0.89 |
| 3.6 | 1.19 | 1.19 | 1.20 | 1.07 | 1.04 |
| 4.6 | 1.21 | 1.18 | 1.20 | 1.13 | 1.10 |
| 4.7 | 1.23 | 1.13 | 1.15 | 1.08 | 1.03 |
| 5.4 | 1.17 | 1.13 | 1.16 | 1.02 | 1.11 |
| 6.6 | 1.00 | 1.23 | 1.10 | 0.86 | 1.03 |
| 7.0 | 0.69 | 1.14 | 0.96 | 0.55 | 0.83 |
| 7.4 | 0.52 | 1.08 | 0.80 | 0.44 | 0.69 |
| 7.6 | 0.25 | 1.06 | 0.62 | 0.23 | 0.54 |
| 7.7 | 0.29 | 1.07 | 0.64 | 0.26 | 0.54 |
| 8.1 | 0.17 | 0.84 | 0.30 | 0.14 | 0.26 |
| 8.5 | 0.0 | 0.68 | 0.19 | 0.0 | 0.19 |
| 9.0 | 0.0 | 0.29 | 0.0 | 0.0 | 0.0 |

[a] $M$ = peak intensity with decoupler power into the $H_2O$ resonance, about 1000 Hz above the amide resonances; $M_0$ = intensity with the decoupler 2000 Hz below the $H_2O$ line.

slopes of plots of $1/T_1$ vs. nitroxyl concentration.

*Calculation of Predicted Exchange Rates.* Predicted exchange rates were calculated for *cyclo*-(Met-Val-Gly-Pro-Asn-Gly)$_2$, and for peptides studied by others, by using the correlation of Molday et al. (1972):

$$\log (k/k_0) = \rho + \lambda$$

Experimental values for $\rho$ and $\lambda$ are available for only a limited number of cases, but on the assumption that they express primarily polar effects, the reported values for Ala were proposed for Met, Val, leu, Ile, and Pro; those for Ser were proposed also the Thr and Cys and those for Tyr also for Phe (Molday et al., 1972).

The reference substance for our calculations was taken to be poly(DL-Ala) since its exchange rates, and their temperature dependence, in water have been determined for both acid and base catalysis (Englander & Poulsen, 1969). For comparison with our observed rate constants or with values reported in the literature, the predicted constants were calculated for the temperature of the experiment, by using the values of $E_{actn}$ determined for poly(DL-Ala) and the appropriate values of the temperature-dependent autoprotolysis constant of water (Bjerrum, 1929).

## Results

Because *cyclo*-(Met-Val-Gly-Pro-Asn-Gly)$_2$ contains no ionizable groups, conformational sensitivity to pH is not expected; in agreement, the chemical shifts of the conformation-sensitive N–H resonances were constant to within 0.01 ppm over the pH range studied, at the fixed temperature of the rate runs. Conformational stability was also indicated by the observation that those resonances of $\alpha$-protons observable in $H_2O$ solution (Pro, Val, plus three protons) showed no significant change in chemical shift over the temperature range 11–55 °C. The maximum shift was 0.05 ppm for the Val $\alpha$-proton.

*cyclo*-(Met-Val-Gly-Pro-Asn-Gly)$_2$ also does not show evidence of aggregation that could affect the observed exchange rates under the conditions of the study: The NMR lines are not noticeably narrowed with increasing temperature, and spectra at 25 mM have no broader lines than those at 3 mM and show the same chemical shifts. At about 25 mM, the peptide elutes from Bio-Gel P-4 at a volume corresponding to its molecular weight.

Table III: Rate Constants for Exchange and Nitroxyl-Induced Relaxation of N-H Protons of *cyclo*-(Met-Val-Gly-Pro-Gly)$_2$ at 21 °C

| N-H unit | $k_{OH^-}$ ($M^{-1}$ $s^{-1}$) | $k_H$ ($M^{-1}$ $s^{-1}$) | $k_{aq}$ ($s^{-1}$) | $k_{nitroxyl}$ ($M^{-1}$ $s^{-1}$) |
|---|---|---|---|---|
| Met | $0.073 \times 10^8$ | $39 \times 10^{-3}$ | $6 \times 10^{-5}$ | 210 |
| Val | $0.34 \times 10^8$ | $20 \times 10^{-3}$ | $6 \times 10^{-5}$ | 410 |
| Gly-3 | $0.012 \times 10^8$ | $34 \times 10^{-3}$ | $5 \times 10^{-5}$ | 160 |
| Asn | $4.7 \times 10^8$ | | | |
| Gly-6 | $0.084 \times 10^8$ | $17 \times 10^{-3}$ | $10 \times 10^{-5}$ | |
| $NH_2$(E) | $0.41 \times 10^8$ | 320 | | 550 |
| $NH_2$(Z) | $0.13 \times 10^8$ | 360 | | 730 |

Experiments at pH 1.9 in which the NaCl concentration was increased to 50 mM from the standard 20 mM value, and experiments at pH 7.1 with the salt concentration set at 100 mM, gave pseudo-first-order rate constants that did not deviate from the pH-dependence lines established for the experiments under standard conditions. Salt effects on the exchange are therefore unimportant here.

General base catalysis was also shown to be unimportant, since rate constants determined in solutions containing malonate ($pK_a$ = 2.8, 5.7) joined without deviation those obtained from solutions buffered with borate ($pK_a$ = 9.1) in the plots of pseudo-first-order rate constant vs. pH.

The absence of salt effects for peptides without ionizable groups (Kim & Baldwin, 1982a,b) and the absence of general base catalysis (Englander et al., 1972)) have been noted earlier by others.

The first-order rate constants for proton exchange are plotted vs. pH in Figure 3A–F, and the rate constants for the separate contributions to the total rate

$$k_{obsd} = k_{aq} + k_{OH}[OH^-] + k_H[H_3O^+]$$

are presented in Table III, along with the measured second-order rate constants for $T_1$ relaxation induced by 2,2,5,5-tetramethylpiperidinyl-1-oxy (Kopple, 1983).

The obvious points of the data in Table III are these: The rate constants for the base-catalyzed reaction differ among themselves by as much as a factor of several hundred, and the variation is roughly in accord with the exposure of the N–H to nitroxyl indicated by the nitroxyl-induced relaxation rate. The rate constants for the acid-catalyzed reaction of the backbone N–H protons only vary by a factor of 2, and they do not obviously correlate with the nitroxyl rate or reflect exposure of the Val N–H proton. Strikingly, there is a factor of $10^4$ difference between the acid-catalyzed rates for the primary amide protons and the backbone protons; in contrast, the base-catalyzed rates for the side chain amide protons are in the range of the backbone protons.

## Discussion

*Observed vs. Predicted Rate Constants. (A) Literature.* What are probably chiefly polar effects of side chains on acid- and base-catalyzed exchange of peptide protons may be estimated by using the linear free-energy relationship proposed by Molday et al. (1972):

$$\log (k/k_0) = \rho + \lambda$$

where $\rho$ and $\lambda$ are substituent constants for the side chains

of the residues providing the carbonyl and nitrogen groups, respectively. Values of these constants were determined from rate measurements made on model *N'*-acetyl amino acid *N*-methylamides. In the absence of electrostatic effects from nearby charged groups, deviations from these predicted rates should result from steric factors absent in the models. Steric factors include not only inhibition of the approach by base or proton donor to the appropriate part of the peptide bond but also steric inhibition of solvation of charged intermediates. Some deviations from predicted rates might be expected because of differences in side chain rotamer distributions between the model and the peptide of interest. In sufficiently large peptides and in cyclic peptides, greater differences are expected from the effects of chain folding, either because the chain shields the exchanging proton or because exchange requires disruption of the peptide conformation at some cost is conformational energy.

Before using the relationship above to predict rates to compare with observation in the case of our cyclic dodecapeptide, we reviewed the literature to find cases in which sufficient pH–first-order rate data were collected to yield reliable values of $k_{OH}$ or $k_H$. We recalculated second-order rate constants in a consistent fashion from the pseudo-first-order rates in each case and calculated the predicted rate constants by using poly(DL-Ala) data for $k_0$. The examples included the acyclic cases of H-Tyr-Asn-Ile-Gln-Lys-OH ( a fragment of ubiquitin) (Krishna et al., 1981), angiotensin II (Lenkinski et al., 1981a), [Sar$^1$,Ala$^8$]angiotensin II, (Asn$^1$,Val$^5$]angiotensin II, and des[Asp$^1$]angiotensin II (Lenkinski et al., 1981b). For the 12 residues excluding tyrosines for which data could be analyzed, the observed second-order rate constants for the base-catalyzed exchange were 0.5–2.0 times that predicted (for four Tyr N–H's in these data, the range was 2.3–3.7 times that predicted). Data for the cyclic peptides tocinoic acid (Sarathy et al., 1981) and [Glu$^4$]oxytocin (Walter et al., 1980) gave an observed/predicted range of 0.1–2.1 for the base-catalyzed process in eight residues. In other cases of the same ring system (Krishna et al., 1979), where only one pH point was available, the observed rate constants were still within a factor of 10 of the predicted values.

On the other hand, for gramicidin S (Philson & Bothner-By, 1979), which is generally considered to have a rigid backbone, the observed/predicted ratio ranged from 1 and 0.1, respectively, for the exposed Phe and Orn N–H protons to 0.0038 and 0.00064, respectively, for the sequestered Leu and Val N–H's, rate inhibitions for base catlaysis of 300× and 1500×. [The gramicidin S data were measured by using 28% (v/v) dioxane, but since base-catalyzed exchange of *N*-methylacetamide is only slowed by a factor of 2 in 50% dioxane (Leichtling & Klotz, 1966), the solvent effect can be taken to be small. Both acid- and base-catalyzed exchange rates for [2,2′-$N^\delta$,$N^\delta$,$N^\delta$-trimethylornithyl]gramicidin S in water were recently reported, and they are not very different from the gramicidin S data (Krauss & Chan, 1982).] Limited data for the probably even more rigid chelated cyclic peptide alumichrome (Llinas et al., 1973) show inhibitions of 3000× or more for the buried N–H protons.

For the acid-catalyzed exchange process, there are relatively few data reported from which second-order rate constants may be extracted. Single pH measurements for angiotensin analogues (Bleich et al., 1973) show acid-catalyzed rates 0.3–2.5 times that predicted, but for gramicidin S, where several data points are available for the acid leg, the second-order rate constants are ca. 0.1 times those predicted for the exposed protons and ca. 0.003 times those predicted for the buried protons.

Table IV: Ratio of Observed to Predicted Second-Order Rate Constants for Proton Exchange in Two Cyclic Peptides[a]

| proton | base catalysis | acid catalysis |
|---|---|---|
| *cyclo*-(Met-Val-Gly-Pro-Asn-Gly)$_2$ | | |
| Met | 0.049 | 0.07 |
| Val | 0.45 | 0.05 |
| Gly-3 | 0.0057 | 0.10 |
| Asn | 2.5 | |
| Gly-6 | 0.014 | 0.06 |
| $NH_2$(E)[b] | 0.48 | 0.5 |
| $NH_2$(Z)[b] | 0.65 | 0.6 |
| *cyclo*-(Val-Orn-Leu-D-Phe-Pro)$_2$[c] | | |
| Val | 0.00064 | 0.0025 |
| Orn | 0.098 | 0.10 |
| Leu | 0.0038 | 0.0031 |
| D-Phe | 1.0 | 0.084 |

[a] Measured values of $\rho$ and $\lambda$ were substituted as follows for the residues for which experimental values have not appeared: Ala for Met, Leu, and Val; Tyr for Phe; and Lys for Orn. [b] Comparison is with experimental rate constants for Ac-Asn-NHMe. [c] Rate constants obtained directly from the data of Philson & Bothner-By (1979); predicted rates calculated as described under Experimental Procedures.

(*B*) *cyclo*-(*Met-Val-Gly-Pro-Asn-Gly*)$_2$. Table IV gives the observed/predicted ratios for *cyclo*-(Met-Val-Gly-Pro-Asn-Gly)$_2$ and those we obtained from the data of Philson & Bothner-By (1979) on gramicidin S.

The rate constants for the primary amide protons of *cyclo*-(Met-Val-Gly-Pro-Asn-Gly)$_2$ are in agreement with those of the study of Ac-Asn-NHMe, Ac-Gln-NHMe, and Ac-Gly-$NH_2$ by Krishna et al. (1982). The proton trans to the carbonyl oxygen exchanges faster in base than does the cis proton, as expected on the basis of lone-pair repulsions in the intermediate amide ion. In acid, the two protons exchange at about the same rate; consistent with exchange by N-protonation as the predominant pathway (Perrin & Johnston, 1981). The fact that all four observed primary amide rate constants are close to 0.5 times the values observed for the corresponding *N'*-acetyl *N*-methylamide indicates that there are negligible steric effects on the primary amide exchange in the cyclic dodecapeptide.

The rate constants for base-catalyzed exchange of the backbone protons in *cyclo*-(Met-Val-Gly-Pro-Asn-Gly)$_2$ show reductions from the rates predicted for exposed protons from the corresponding *N*-acetyl *N'*-methylamides in agreement with the proposed conformation. This adds support both to the hypothesis that base-catalyzed exchange is sensitive to solvent exposure and to the proposed conformation itself. In the model shown in Figure 1, the Val and Asn backbone N–H's are exposed to solvent, and their second-order rate constants for base-catalyzed exchanged are within a factor of 3 of those predicted, good agreement considering the results of the literature survey. On the other hand, the Met and both Gly backbone N–H's are directed into the ring and show 20–180-fold inhibitions relative to the calculated estimates. While these inhibitions are not as large as those observed for the Leu and Val protons of gramicidin S, they are significant. They correlate, where the data are available, with the estimates of solvent exposure given by second-order rate constants for relaxation by 2,2,6,6-tetramethylpiperidinyl-1-oxy [see Table III and the data in Kopple & Go (1977)]. Quantitative data for nitroxyl relaxation of gramicidin S are published for dimethyl sulfoxide solutions only (Niccolai et al., 1982), but they correlate with the exchange rates in water also, consistent with

the generally accepted hypothesis that the backbone of gramicidin S is solvent independent.

*Solvent-Induced Overhauser Enhancements and Solvent Exposure.* The data in Table II suggest an additional method for determining the solvent exposure of peptide backbone protons. In the pH region where exchange does not contribute to the $T_1$ relaxation rate, positive nuclear Overhauser enhancements of the Met, Val, and Gly-3 N–H resonances are produced on irradiating the solvent. From eight or nine measurements in each case, these enhancements are about 12% (±3) for Met, 23% (±1.3) for Val, and 15% (±4) for Gly-3, where the standard deviations are given in parentheses. To the extent that their magnitudes reflect the contribution of the water protons to the relaxation of the N–H protons, the enhancements reflect the average exposure of the N–H protons to the solvent. Since other protons of the peptide also contribute to the relaxation, the solvent-induced NOE may not be a reliable measure of solvent exposure, but it is of interest to note that the average solvent-induced NOE's are distinctly less for the sequestered Met and Gly-3 protons than for the exposed Val N–H.

*Base-Catalyzed Exchange.* It is not established whether the observed inhibitions of base-catalyzed exchange measure the extent of solvent exposure of N–H's in a completely rigid peptide or are related to the ratio of rapidly exchanging populations in which N–H's are exposed or buried. More likely the inhibitions reflect something in between. Even if there were no librations about a minimum energy conformation, no exchangeable proton in a cyclic oligopeptide is completely buried from the solvent. Gramicidin S and *cyclo*-(Met-Val-Gly-Pro-Asn-Gly)$_2$ are to a first approximation flat rings, with the solvent-shielded exchangeable protons still exposed from above and below the ring planes. It is likely that exchange of these sequestered protons can occur without gross disruption of the preferred conformations, although there will be a contribution to the activation barrier from whatever distortion is necessary. The linear dependence of the first-order exchange rates on $[H^+]$ and $[OH^-]$ between $10^{-4}$ and $10^2$ s$^{-1}$ does show that for *cyclo*-(Met-Val-Gly-Pro-Asn-Gly)$_2$ the kinetic barrier to proton exchange is not a conformational change occurring in that frequency range.

*Mechanism of Acid-Catalyzed Exchange.* Considering the roughly planar form of the cyclic peptide rings, it seems that in exchange by N-protonation approach of the proton donor from one side or the other of the trigonal nitrogen should not be highly dependent on the details of the chain folding. In the base-catalyzed process, in contrast, the base must approach the exchanging proton approximately along the N–H bond direction where interference by the backbone is likely to be great if the N–H bond is internally directed. N-Protonation for acid catalysis is consistent with the lack of correlation in the cyclic dodecapeptide between the observed/predicted ratios for acid catalysis and those for base catalysis. Although the base-catalyzed rate constants of the backbone protons have a range of 400, the acid-catalyzed rates only vary within a factor of 2. For gramicidin S, the division into solvent-shielded Leu and Val protons and exposed Orn and Phe protons is retained in acid, but the rate range is smaller, 40-fold, than in base, 1500-fold.

In the cyclic dodecapeptide, the acid-catalyzed rates for the backbone protons are 10–20 times slower than those predicted. In gramicidin S, they are 10–400 times slower. Only limited data for the acid side of the pH–rate profiles of acyclic peptides are available for comparison. However, for two angiotensin analogues, [Asn$^1$,Val$^3$]angiotensin II at pH 2.3 and H-Val-Tyr-Val-His-Pro-Phe-OH at pH 1.6, well in the acid-catalyzed region (Bleich et al., 1973), we calculated the exchange rates for eight exchangeable protons to be within a factor of 2.5 of those estimated by using the parameters of Molday et al. (1972). In the cyclic peptides, there therefore appears to be a distinct inhibition of acid-catalyzed exchange even for solvent-exposed backbone N–H protons. It seems reasonable to associate the inhibition of the acid-catalyzed process with resistance of the cyclic backbone to distortion. An N-protonation mechanism requires that the C′–N–$C_\alpha$ backbone bond angle change from trigonal to tetrahedral. The more stable the backbone conformation, the more this change will be resisted, and the greater the reduction in rate relative to less constrained peptides. It would be of interest to have additional precise measurements for acid-catalyzed exchange, to test whether it is really a measure of conformational rigidity. Cyclic pentapeptides might provide good models for the purpose, since it has been suggested that these have large energy barriers to distortion at nitrogen (C. Ramakrishnan, unpublished results).

Acid-catalyzed exchange by an O-protonation mechanism is probably not the dominant path for peptides (Perrin & Johnston, 1981). The results presented here also support N-protonation. O-Protonation does not result in a change in bond angles and thus provides no ready rationale for the difference between cyclic and acyclic peptides. It might also be expected that exchange catalyzed by O-protonation could be inhibited when the C=O of the peptide bond is directed into the ring, as is the case for the Met-Val unit in the cyclic dodecapeptide and the Leu-Phe and Val-Orn units in gramicidin S. There is no significant indication of such inhibition.

*Summary.* In base-catalyzed exchange in both cyclic peptides, the important factor is the direction of the N–H bond relative to the ring. Direction into the ring (solvent shielding) leads to inhibition of exchange rates. However, base-catalyzed proton exchange rates or the observed to predicted rate ratios probably cannot be used as criteria of solvent accessibility unless there is other evidence that a single conformation is dominant and unless electrostatic contributions are assuredly negligible. For acid catalysis, the gramicidin S data show that exposure of N–H is more important than exposure of C=O, but the *cyclo*-(Met-Val-Gly-Pro-Asn-Gly)$_2$ results show no relation between conformation and exchange rate. An N-protonation mechanism fits the fact that in both cyclic peptides all of the protons exchange more slowly than analogously situated peptide protons in less conformationally constrained acyclic cases.

**Registry No.** *cyclo*-(Met-Val-Gly-Pro-Asn-Gly)$_2$, 65991-00-6; *cyclo*-(Val-Orn-Leu-D-Phe-Pro)$_2$, 113-73-5.

## References


Bjerrum, N. (1929) *International Critical Tables* (Washburn, E. W., Ed.) Vol. VI, p 152, McGraw-Hill, New York.

Bleich, H. E., Galardy, R. E., Printz, M. P., & Craig, L. C. (1973) *Biochemistry 12*, 4950–4957.

Englander, S. W. (1975) *Ann. N.Y. Acad. Sci. 244*, 10–27.

Englander, S. W., & Poulsen, A. (1969) *Biopolymers 7*, 379–393.

Englander, S. W., Downer, N. W., & Teitelbaum, H. (1972) *Annu. Rev. Biochem. 41*, 903–924.

Hvidt, A., & Nielsen, S. O. (1960) *Adv. Protein Chem. 21*, 287–386.

Kim, P. S., & Baldwin, R. I. (1982a) *Biochemistry 21*, 1–5.

Kim, P. S., & Baldwin, R. I. (1982b) *Biochemistry 21*, 3036.

Kopple, K. D. (1983) *Int. J. Pept. Protein Res. 21*, 43–48.

Kopple, K. D., & Go, A. (1977) *J. Am. Chem. Soc. 99*, 7698–7704.
Krauss, E. M., & Chan, S. I. (1982) *J. Am. Chem. Soc. 104*, 6953–6961.
Krishna, N. R., Huang, D. H., Glickson, J. D., Rowan, R., & Walter, R. (1979) *Biophys. J. 26*, 345–366.
Krishna, N. R., Goldstein, S., & Glickson, J. D. (1980) *Biopolymers 19*, 2009–2020.
Krishna, N. R., Huang, D. H., Vaughn, J. B., Heavner, G. S., & Goldstein, G. (1981) *Biochemistry 20*, 3933–3940.
Krishna, N. R., Sarathy, K. P., Huang, D. H., Stephens, R. L., Glickson, J. D., Smith, C. W., & Walter, R. (1982) *J. Am. Chem. Soc. 104*, 5051–5053.
Leichtling, B. J., & Klotz, I. M. (1966) *Biochemistry 5*, 4026–4037.
Lenkinski, R. E., Stephens, R. L., & Krishna, N. R. (1981a) *Biochemistry 20*, 3122–3126.
Lenkinski, R. E., Stephens, R. L., & Krishna, N. R. (1981b) *Biochim. Biophys. Acta 667*, 157–167.
Llinas, M., Klein, M. P., & Nielands, J. B. (1973) *J. Biol. Chem. 248*, 915–923.
Madison, V. (1978) *Biopolymers 17*, 1601–1604.
Molday, R. S., Englander, S. W., & Kallen, R. G. (1972) *Biochemistry 11*, 150–158.
Narutis, V. P. (1983) Ph.D. Thesis, Illinois Institute of Technology, Chicago, IL.
Niccolai, N., Valensin, G., Rossi, C., & Gibbons, W. A. (1982) *J. Am. Chem. Soc. 104*, 1534–1537.
Perrin, C. L., & Johnston, E. R. (1981) *J. Am. Chem. Soc. 103*, 4697–4703.
Philson, S. B., & Bothner-By, A. A. (1979) *Proceedings of the American Peptide Symposium, 6th* (Gross, E., & Meienhofer, J., Eds.) pp 219–222, Pierce Chemical Co., Rockford, IL.
Redfield, A. G. (1978) *Methods Enzymol. 49*, 253–269.
Redfield, A. G., Kunz, S. D., & Ralph, E. K. (1975) *J. Magn. Reson. 19*, 114–117.
Sarathy, K. P., Krishna, N. R., Lenkinski, R. E., Glickson, J. D., Rockway, T. W., Hruby, V. J., & Walter, R. (1981) *Neurohypophyseal Peptide Hormones and Other Biologically Active Peptides* (Schlesinger, D. H., Ed.) pp 211–226, Elsevier/North-Holland, New York.
Walter, R., Smith, C. W., Sarathy, K. P., Pillai, R. P., Krishna, N. R., Lenkinski, R. E., Glickson, J. D., & Hruby, V. J. (1980) *Int. J. Pept. Protein Res. 17*, 56–64.
Woodward, C. K., & Hilton, B. D. (1979) *Annu. Rev. Biophys. Bioeng. 8*, 99–127.

# Proton Nuclear Magnetic Resonance Investigation of the Conformation-Dependent Spin Equilibrium in Azide-Ligated Monomeric Insect Hemoglobins†

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ABSTRACT: The proton nuclear magnetic resonance spectra of the met–azide complexes of the two allosteric monomeric hemoglobins of the insect larva *Chironomus thummi thummi* have been recorded, assigned, and analyzed. Both the magnitude of the heme methyl shifts and their anomalous temperature dependence indicate a rapid equilibrium between a low-spin ($S = {}^1/_2$) and a high-spin ($S = {}^5/_2$) state. Using the mean methyl hyperfine shift as an indicator of the position of the spin equilibrium, we demonstrate that the axial ligand field is influenced by the heme orientational position in the heme cavity, by the protein conformational state for each heme orientation, and by the presence of a silent point mutation in the heme cavity. The proximal histidyl imidazole exchangeable protons are assigned for the met–azide complexes in both the *Chironomus* hemoglobin and sperm whale myoglobin, and their magnitude reflects a similar percent high-spin component as that derived from the mean heme methyl shift. The pH dependence of the hyperfine shifts reflects a p$K$ consistent with the Bohr effect. The change in percent high spin in the t $\rightleftharpoons$ r transition is found to be too small to account for the Bohr effect. The difference in the position of the equilibrium for the two heme orientations, however, suggests that the two compounds may exhibit different amplitudes of the Bohr effect.

The monomeric hemoglobins from insect larva of *Chironomus thummi thummi* provide ideal subjects for investigating the allosteric control of dioxygen binding. Two of these $O_2$-binding proteins, labeled CTT III and CTT IV, exhibit marked alkaline Bohr effects in spite of remaining monomeric over the range of pH 5–10, where these proteins are stable in their native form (Sick & Gersonde, 1969; Gersonde et al., 1972, 1976). The Bohr effect is modulated by the particular exogenous ligand bound to the sixth coordination site of the heme iron and is largest for $O_2$ and considerably smaller for CO (Gersonde et al., 1976; Trittelvitz et al., 1973; Sick & Gersonde, 1974; Steffens et al., 1977). On the other hand, neither the oxidation state of the heme iron (La Mar et al., 1978a) nor the substitution of iron with cobalt (Gersonde et al., 1982) appears to have a significant influence on the Bohr effect. Furthermore, the exchange of proto- for meso- or deuteroporphyrin does not influence the magnitude of the Bohr effect in these hemoglobins (La Mar et al., 1978a; Gersonde et al., 1982). The tertiary structural transition responsible for the Bohr effect is controlled by a single proton, i.e., the formation of a C-terminal salt bridge (Sick et al., 1972; Ribbing & Ruterjans, 1980a), appearing in both the unligated (deoxy) and all ligated states (Trittelvitz et al., 1973; La Mar et al.,

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0006-2960/83/0422-6239$01.50/0 