Conformational Diversity of Bradykinin in Aqueous Solution†

L. Denys, A. A. Bothner-By,* G. H. Fisher, and J. W. Ryan

ABSTRACT: The 600-MHz proton nuclear magnetic resonance spectra of bradykinin, [2-dehydroproline]bradykinin, [7-dehydroproline]bradykinin, and [5-tyrosine]bradykinin in aqueous solution have been recorded and completely assigned by means of pH variation, spin–spin decoupling, and chemical shift correlations. Analysis of the spin–spin coupling constants in the main chain and in the side chains suggests that bradykinin is in rapid equilibrium among many conformers and does not show any persistent structural features such as β turns or internal hydrogen bonds. Addition of lipids or lipid-like materials [such as sodium (trimethylsilyl)propionate] in high concentration causes changes in the spectra, indicating specific interactions with proline-7 and phenylalanine-8, as well as a change in side-chain rotameric preference.

Bradykinin (BK)[1] is a nonapeptide hormone (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) with manifold physiological properties. It stimulates the contraction of smooth muscle, both directly and indirectly via the prostaglandins, inhibits neurotransmission in the spinal cord, and causes the release of catecholamines in the adrenal medulla. Centrally administered BK potentiates phenobarbital sleeping time, as does the tripeptide fragment Phe-Ser-Pro.

Bradykinin contains three proline residues, which are often associated with bends in the chain direction in proteins, and it might be expected that this could produce a favored solution conformation. The Chou & Fasman (1979) probability factor for a β turn is calculated to be 3.79×10^{-4} for the sequence $Pro^2Pro^3Gly^4Phe^5$ and 1.99×10^{-4} for $Ser^6Pro^7Phe^8Arg^9$. Both of these factors are high enough to indicate a very probable β turn if these sequences are found in a protein.

Cann and co-workers in a series of papers (Cann, 1972; Cann et al., 1973, 1976, 1979; London, 1979) have reported on a study of the CD of BK and fragments thereof, as well as of analogues, and have concluded that BK exists largely in a disordered state but that a small fraction (<20%) may exist in a form with a γ turn bridging Pro^7. The CD of BK and analogues of BK in which the aromatic residues Phe^5 and Phe^8 have been replaced was studied by Lintner et al. (1977), who concluded that Phe^5 and Phe^8 contribute differently and are conformationally distinguishable. From changes in the CD spectra between BK and BK(2–9), Lintner et al. (1979) concluded that a β turn could be present in the sequence Pro-Pro-Gly-Phe.

London et al. (1978) have recorded and assigned the ^{13}C spectra of BK and deduced that the prolines are predominantly trans (≥90%) and that all side chains were in rapid rotation. The solvent dependence of the Ser^6 carbonyl resonance suggested that Ser^6 is involved in an internal H bond. Lintner & Fermandjian (1979) have observed and partially assigned the 250-MHz proton spectrum of BK. They note that titration of Arg^9 causes shifts in Phe^8, Arg^9, and Gly^4.

In this work, we have attempted to obtain further information on the conformational states of BK in aqueous solution, by using 600-MHz proton NMR spectroscopy. To this end, we have recorded the spectra of BK and several analogues of BK, [2-(3,4-dehydroproline)]bradykinin, [7-(3,4-dehydroproline)]bradykinin, and Tyr^5-BK, observing the effect of pH variation. Complete assignments of the spectra were made, and extensive spin–spin analysis has been performed, aided by observation of the spectra of [2-(3,4-dideuterioproline)]-bradykinin and [7-(3,4-dideuterioproline)]bradykinin. We have determined the rate of base-catalyzed exchange of the amide protons and have observed the effects of addition of lipids to aqueous solutions of BK on the proton spectra.

Materials and Methods

Bradykinin and Tyr^5-BK. Several samples were purchased from U.S. Biochemicals. They were used without further purification, since no minor constituents could be detected by NMR spectroscopy.

[2-(3,4-Dehydroproline)]bradykinin and [7-(3,4-Dehydroproline)]bradykinin. These materials were synthesized as described previously (Fisher et al., 1978).

[2-(3,4-Dideuterioproline)]bradykinin and [7-(3,4-Dideuterioproline)]bradykinin. The corresponding (3,4-dehydroproline)bradykinin (5 mg) was dissolved in 2.5 mL of ^2H_2O, and 5 mg of palladium black was added. The mixture was stirred under 1 atm pressure of $^2H_2$ gas (Matheson) for several hours, after which the solution was filtered and concentrated. The NMR spectrum indicated complete hydrogenation (no olefinic proton signals detectable), and the reaction appeared to be highly stereospecific (the β'-proton signal was reduced in relative intensity compared to the parent BK by ~90%).

NMR Spectroscopy. Measurements were all performed by using the 600-MHz NMR spectrometer at the NMR Facility for Biomedical Studies in the rapid-scan correlation mode in order to suppress the water peak. ^2H_2O was used as a solvent in all cases except when the amide proton signals were to be observed, in which case a mixture of 60% H_2O and 40% ^2H_2O was used. For observation of signals close to the water peak, such as that from the α proton of proline-2, the water signal was inverted by a selective soft pulse, and the scan was then performed on a schedule such that the water signal was near zero intensity when traversed. The amide signals were assigned by underwater decoupling experiments, as described by Dadok et al. (1972).

The pH was adjusted by addition of NaO^2H or ^2HCl. Values quoted are uncorrected pH meter readings. Chemical shifts are quoted relative to the (trimethylsilyl)propionate signal.

† From the Department of Chemistry, Carnegie-Mellon University, Pittsburgh, Pennsylvania 15213 (L.D. and A.A.B.), and the Department of Medicine, University of Miami School of Medicine, Miami, Florida 33101 (G.H.F.). *Received June 29, 1982.* This work was supported by Grants AM16532 and HL 22896-04 from the National Institutes of Health and was performed at the NMR Facility for Biomedical Studies, supported by National Institutes of Health Grant RR00292.

[1] Abbreviations: BK, bradykinin; CD, circular dichroism; NMR, nuclear magnetic resonance; $NaDodSO_4$, sodium dodecyl sulfate; TSP, tetradeuterio(trimethylsilyl)propionate.

0006-2960/82/0421-6531$01.25/0

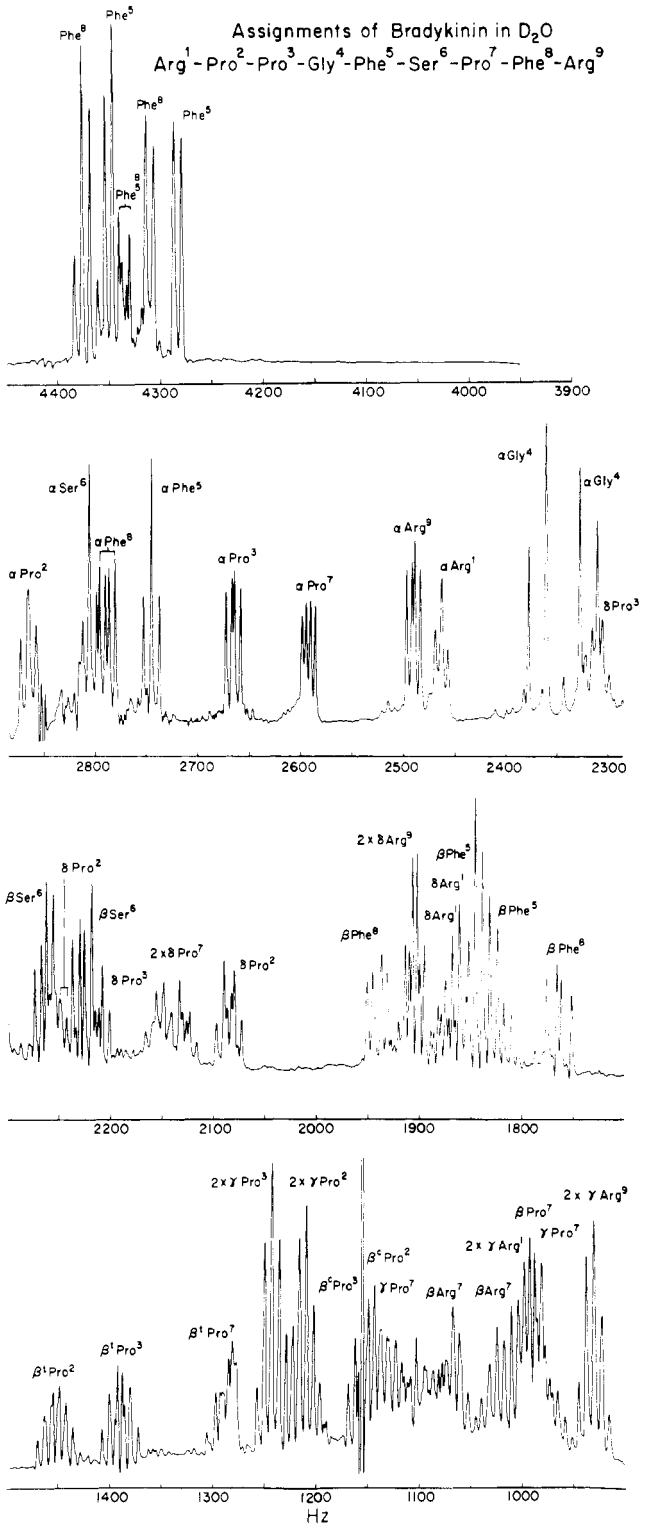

FIGURE 1: ^1H NMR spectrum of bradykinin at 600 MHz, 10 mM in ^2H_2O, pH 7.4, showing all assignments (see text).

Spectra were simulated by using the LAOCN3 program (Castellano & Bothner-By, 1964) and plotted by using the ITRCAL program on an Aspect 2000. In some cases (e.g., overlapping β-Pro and β-Arg resonances), difference spectra were used to obtain the decoupling patterns suggested by spectral simulation.

Results

Assignments. The spectrum of BK and the assignments deduced for it are shown in Figure 1. Table I contains the shifts and coupling constants evaluated. Assignments generally are based on a combination of comparison of shifts with values expected from the literature (Bundi & Wuethrich, 1979), effects of pH titration (Wuethrich, 1976), and spin decoupling. Thus, the α-proton signals of Arg^1 and Arg^9 were identified by (1) their position in the spectral region appropriate for α protons, (2) their titration behavior, consistent with the pK values reported by Paiva & Juliano (1977) for the amino and

Table I: Measured Shifts and Coupling Constants of Bradykinin, 10 mM in ^2H_2O at pH 7.4, 28 °C

residue	proton[a]	chemical shift (ppm) relative to TSP	coupling constants determined
Arg^1	α	4.105	$J_{\alpha\beta} + J_{\alpha\beta'} = 12.46$
	β	1.730	
	β'	1.730	
	γ	1.660	
	γ'	1.652	
	δ	3.012	
	δ'	3.085	
Pro^2	α	4.776	$J_{\alpha\beta^c} = 7.2, J_{\alpha\beta^t} = 8.2$
	β^c	1.890	$J_{\beta^c\beta^t} = -12.6$
	β^t	2.420	$J_{\beta^c\gamma^t} = J_{\beta^t\gamma^c} = 6.6$
	γ^c	2.015	$J_{\beta^c\gamma^c} = J_{\beta^t\gamma^t} = 7.0$
	γ^t	2.027	$J_{\gamma^t\delta^c} = J_{\gamma^c\delta^t} = 6.2$
	δ^c	3.752	$J_{\gamma^c\delta^c} = J_{\gamma^t\delta^t} = 7.1$
	δ^t	3.474	$J_{\delta^t\delta^c} = -10.2$
Pro^3	α	4.443	$J_{\alpha\beta^c} = 5.8, J_{\alpha\beta^t} = 8.4$[d]
	β^c	1.920	$J_{\beta^c\beta^t} = -12.9$
	β^t	2.315	$J_{\beta^c\gamma^c} = J_{\beta^t\gamma^t} = 7.8$
	γ^c γ^t	2.070	$J_{\beta^c\gamma^t} = 5.4, J_{\beta^t\gamma^c} = 6.8$
	δ^c	3.851	
	δ^t	3.689	
Gly^4	α	3.947	$J_{\alpha\alpha'} = 15.6$
	α	3.867	
Phe^5	α	4.574	$J_{\alpha\beta(S)} = 6.29$
	$\beta(S)$	3.074	$J_{\alpha\beta(R)} = 7.43$
	$\beta(R)$	3.034	$J_{\beta\beta} = -13.83$
	δ	7.230	
	ϵ	7.352	
	ζ	7.31[b]	
Ser^6	α	4.677	$J_{\alpha\beta} = 6.38$
	β	3.771	$J_{\alpha\beta} = 7.43$
	β	3.717	$J_{\beta\beta} = -11.40$
Pro^7	α	4.321	$J_{\alpha\beta^c} = 4.3, J_{\alpha\beta^t} = 8.7$
	β^c	1.685[c]	$J_{\gamma^c\delta^c} = J_{\gamma^t\delta^t} = 7.2$
	β^t	2.151	$J_{\gamma^c\delta^t} = 6.5, J_{\gamma^t\delta^c} = 7.4$
	γ^c	1.683	$J_{\delta^c\delta^t} = -10.9$
	γ^t	1.90	
	δ^c	3.586	
	δ^t	3.547	
Phe^8	α	4.648	$J_{\alpha\beta(S)} = 5.61$
	$\beta(S)$	3.232	$J_{\alpha\beta(R)} = 9.71$
	$\beta(R)$	2.935	$J_{\beta\beta} = -13.99$
	δ	7.280	
	ϵ	7.390	
	ζ	7.31[b]	
Arg^9	α	4.150	$J_{\alpha\beta(S)} = 5.15$
	β	1.830	$J_{\alpha\beta(R)} = 8.11$
	β	1.702	
	γ	1.551	
	γ	1.551	
	δ	3.160	
	δ	3.180	

[a] In proline residues, superscripts c and t refer to protons cis and trans to the proline carboxyl group. [b] Para protons of Phe^5 and Phe^8 overlap. [c] The β^c proton in Pro^7 is accidentally degenerate with one of the γ protons. [d] For evaluation of the coupling constants in proline, the rules developed by Bach et al. (1982) were used.

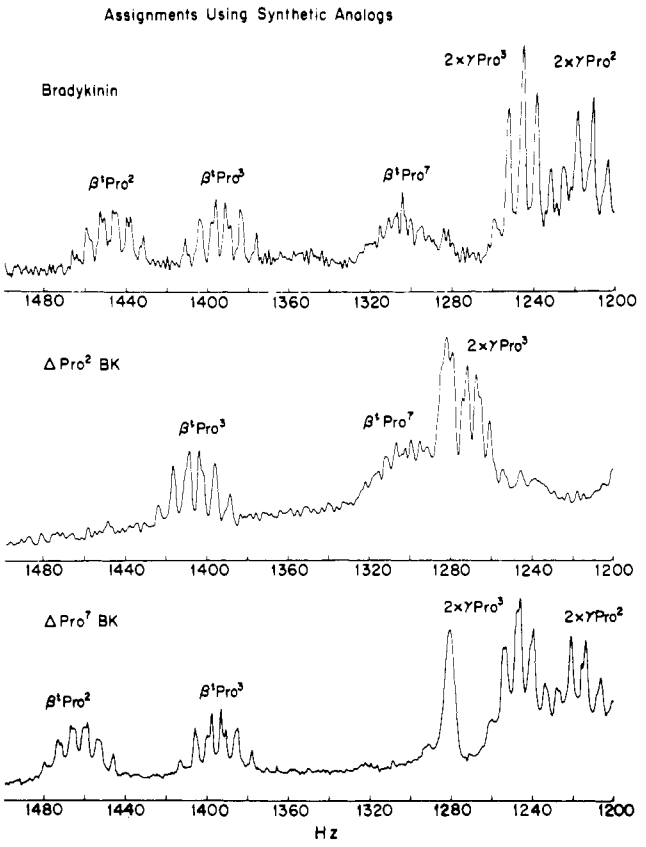

FIGURE 2: Comparison of a portion of the spectra of bradykinin, [2-(3,4-dehydroproline)]bradykinin and [7-(3,4-dehydroproline)]-bradykinin, allowing assignment of the β' proton resonances as well as the γ resonances of proline-2.

carboxyl groups of BK, and (3) decoupling from the β protons, which were thereby located in the spectral region appropriate for them.

For assignment of the proline resonances to the correct prolines, a comparison of the spectra of BK, [2-(3,4-dehydroproline)]bradykinin, and [7-(3,4-dehydroproline)]-bradykinin was made. Figure 2 provides a view of the region where the β' protons of the proline are normally observed. It can be seen that one of the β' resonances is missing in each of the dehydroproline analogues, allowing unambiguous assignment of that peak. Similar effects were observed for the α protons and δ protons. These spectral assignments were confirmed on examination of the corresponding dideuterio-proline analogues. Extensive decoupling experiments established the connectivities of α, β, γ, and δ protons in each ring.

The α and β resonances of Phe[5] and Phe[8] were assigned by comparison of the spectra with those of Tyr[5]-BK. Assignments of the Phe[5] and Phe[8] aromatic proton resonances were made in the same way. The assignments agree with those of Lintner & Fermandjian (1979), made on the basis of pH effects.

The Gly[4] resonances were identified as an AB quartet in the correct region. The assignment was confirmed by decoupling from the amide proton, which appeared as a triplet in H_2O solution.

The remaining resonances were those of Ser[6], which were assigned on the basis of decoupling and appearance in the correct spectral region. There is overlap of the serine resonances with those of the δ protons of Pro[2] and Pro[3]. Decoupling was detected by using difference spectroscopy.

Chemical Shifts. Comparison of the chemical shifts of the protons of BK in aqueous solutions at pH 7.4 with those expected for a random coil (Bundi & Wuethrich, 1979) shows

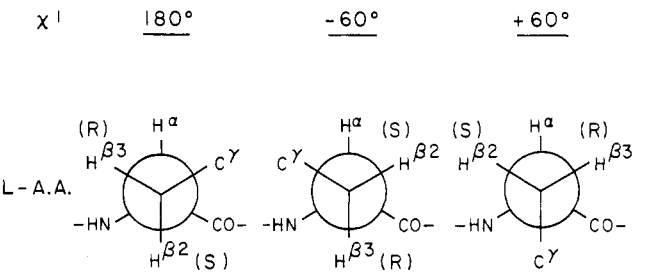

FIGURE 3: Classical rotamer conformations assumed for nonproline residues of bradykinin.

Table II: Populations of Rotamers Deduced for Nonproline Side Chains in Bradykinin

	populations in bradykinin[a]			populations in random coil model[a]		
residue	χ_{180}	χ_{-60}	χ_{+60}	χ_{180}	χ_{-60}	χ_{+60}
Arg[1]	0.66		0.34	0.72		0.28
Arg[9]	0.50	0.23	0.27			
Phe[5]	0.34	0.44	0.22	0.27	0.70	0.03
Phe[8]	0.27	0.65	0.08			
Ser[6]	0.75		0.25	0.5		0.5

[a] Degenerate or unassigned β and β' protons allow evaluation of the sum of populations for χ_{180} and χ_{-60}.

that all fall in the expected ranges with the possible exceptions of the α protons of Pro[2], Phe[5], and Phe[8]. The shifts of α protons, however, are strongly dependent on the identity of the neighboring residues (Wuethrich, 1976), so that no deductions of structure can be made.

One striking observation is that the δ protons of Arg[1] yield signals which are chemically shifted from each other by about 0.06 ppm. This nonequivalence has not been observed before, to our knowledge, and suggests that BK spends at least a part of its time in conformations in which the terminus of the arginine side chain is in a strongly asymmetric environment, perhaps adjacent to the aromatic ring of Phe[5].

Spin–Spin Coupling Constants and Side-Chain Rotamer Populations. The $^3J_{\alpha\beta}$ coupling constants in all nonproline residues were used to deduce rotamer populations for the individual side chains. The conformers assumed are as shown in Figure 3, and the populations were calculated from the following relations:

$$p_{180} = [J_{\alpha\beta(S)} - 2.6]/11.0 \quad (1)$$

$$p_{-60} = [J_{\alpha\beta(R)} - 2.6]/11.0 \quad (2)$$

$$p_{+60} = 1 - p_{180} - p_{-60} \quad (3)$$

which were based on earlier work (Pachler, 1964; Feeney, 1976; Kobayashi & Nagai, 1978). The assignments of *S* and *R* protons were based on previously observed regularities (Kobayashi & Nagai, 1978) but have not been independently verified. The calculated populations are shown in Table II. They agree well with the values obtained by Lintner et al. (1979) at 250 MHz. One may deduce two things from these values. First, the populations are similar to those calculated for the corresponding amino acid residues in random coil models (Bundi & Wuethrich, 1979), also shown in Table II. Thus, none of the side chains are locked into unique rotamers, as might occur if sections of the peptide were rigid. Second, since the distribution into rotamers is different for the different side chains, it appears unlikely that an equilibrium between only two or three conformers with highly constrained side chains occurs. Phe[5] shows slightly less of the χ_{-60} rotamer and slightly more of the χ_{60} rotamer than the random coil model,

Table III: Average Deviation from Planarity,[a] $\hat{\chi}$, and Average Angle of Twist,[b] $\tilde{\chi}$, for Ring Bonds in the Prolines of Bradykinin[c]

residue	$\hat{\chi}_1$	$\tilde{\chi}_1$	$\hat{\chi}_2$	$\tilde{\chi}_2$	$\hat{\chi}_3$	$\tilde{\chi}_3$
Pro[2]	26	6	30		29	1
Pro[3]	23	0				
Pro[7]	21	6			29	2

[a] Defined as $\cos^{-1}\langle\cos\chi\rangle$. [b] Defined as $\sin^{-1}\langle\sin\chi\rangle$. [c] Units are degrees.

Table IV: Shifts and Coupling Constants of Amide Protons in Bradykinin in H_2O at pH 5.0

residue	chemical shift (ppm) relative to TSP	J_{HNCH} (Hz)
Gly[4]	8.42	5.8
Phe[5]	8.03	6.7
Ser[6]	8.15	7.7
Phe[8]	8.01	7.1
Arg[9]	7.72	7.5

but about the same rotamer distribution as that calculated for Gly-Phe or Phe-Gly (Kozlowski et al., 1977). The Ser[6] side chain is calculated to have a population of χ_{60} of only 0.25, half that of the random coil model, and also less than that in other models (Ptak et al., 1978).

Proline Rings. Analysis of the proton spin coupling constants in the three proline rings was complicated by spectral overlap and accidental degeneracies of chemical shifts. By a combination of application of the rules governing coupling constants in proline rings developed by Bach et al. (1982) and examination of the spectra of the dideuterio analogues, it was nevertheless possible to derive a fairly complete set of coupling constants for proline-2 and partial sets for proline-3 and proline-7. The coupling constants obtained have been used to evaluate $\hat{\chi}$ and $\tilde{\chi}$, as presented in Table III. $\hat{\chi}$ and $\tilde{\chi}$ are obtained, respectively, from the mean cos χ and mean sin χ of the ring bonds. The theory for this analysis has been presented by Bach et al. (1982). $\hat{\chi}$ measures the degree of twist in the bond, or nonplanarity of the ring without regard to the direction of twist, while $\tilde{\chi}$ measures the degree of twist taking into account the sign of the angle.

For all three proline rings, the degree of puckering, as given by $\hat{\chi}$, is typical for proline in unconstrained environments. The preference for Ramachandran A or B forms, however, as given by $\tilde{\chi}$ is small (within experimental error of zero) for all three prolines. Thus, it appears that none of the prolines are in a rigid segment of the peptide.

Amide NH Coupling Constants and Exchange Rates. The amide proton resonances are shown with their assignments in Figure 4. Shifts and coupling constants at pH 5.0 are given in Table IV. Variation of pH over the range 2–6 did not cause any measurable changes in the $^3J_{HCNH}$ coupling constants. The magnitude of the coupling constants suggests that the backbone at each of these residues rotates freely and that the rotamer population is very nearly equal for the three rotamers. In Gly[4], the two α protons are equally coupled with the adjacent NH; thus, no evidence for the incorporation of this residue in a β turn is found.

The apparent rate of base-catalyzed exchange of NH with solvent water at 21 °C was determined for each of the amide resonances by raising the pH of the solution to the point where significant exchange broadening could be measured. The exchange rate at this point was deduced from

$$R = \pi(\nu_{1/2} - \nu_{1/2}^0) \quad (4)$$

where $\nu_{1/2}$ is the width at half-height of the broadened line

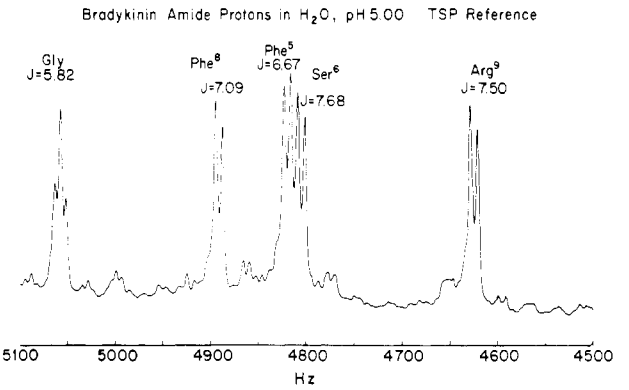

FIGURE 4: Amide proton resonance signals of bradykinin at pH 5.0, with assignments.

Table V: Measured and Predicted[a] Rates for Base-Catalyzed Amide Proton Exchange in Bradykinin

residue	measured $k_{OH} \times 10^8$	predicted $k_{OH} \times 10^8$
Gly[4]	3.1	4.2
Phe[5]	2.3	2.4
Ser[6]	4.3	6.0
Phe[8]	1.7	1.2
Arg[9]	0.66[b]	0.11

[a] Alanine dipeptide was taken as the base rate for calculated values ($k_{OH} = 1.5 \times 10^8$ s^{-1}). [b] The terminal carboxylate decreases the exchange rate by about 20-fold.

and $\nu_{1/2}^0$ is the width at pH 3.0, where exchange broadening is negligible. The rate constant for base-catalyzed exchange, k_{OH}, was then obtained from

$$R = k_{OH}[OH^-] \quad (5)$$

The rate constants obtained are shown in Table V, along with the rate constants calculated for NH protons in random coil models by the method of Molday et al. (1972). Incorporation of any of the amide protons into an internal hydrogen bond is expected to retard the base-catalyzed exchange (Philson & Bothner-By, 1979) by 2–3 orders of magnitude. In the case of BK, however, all rate constants agree to well within 1 order of magnitude, suggesting that internal hydrogen bonds are absent, or nearly so.

Interactions with Lipids. From an examination of spectra of BK solutions containing various amounts of sodium tetradeuterio(trimethylsilyl)propionate (TSP), the reference substance, it was observed that several spectral parameters depended on the concentration of TSP. Addition of inorganic salts did not cause these changes, which therefore appeared to be caused by interaction of BK with the lipid-like anion. The changes were monitored during titration of the solution with TSP, and it was found that many of the resonances were little affected, but that others, especially proton resonances assigned to Phe[5], Ser[6], Pro[7], and Phe[8], shifted according to a sigmoidal curve over the TSP concentration range 0.0–2.0 M. Plots of shifts vs. concentration for all resonances which showed an overall shift of 0.05 ppm or more are shown in Figure 5.

If BK and TSP form a 1:1 complex reversibly, the shift should measure directly the fraction of complex formed, so that

$$K_f = \frac{[BT]}{[B][T]} = \frac{\delta - \delta_B}{\delta_{BT} - \delta}\frac{1}{[T]} \quad (6)$$

where [B], [T], and [BT] represent the concentrations of free BK, free TSP, and BK–TSP complex, respectively, and δ, δ_B,

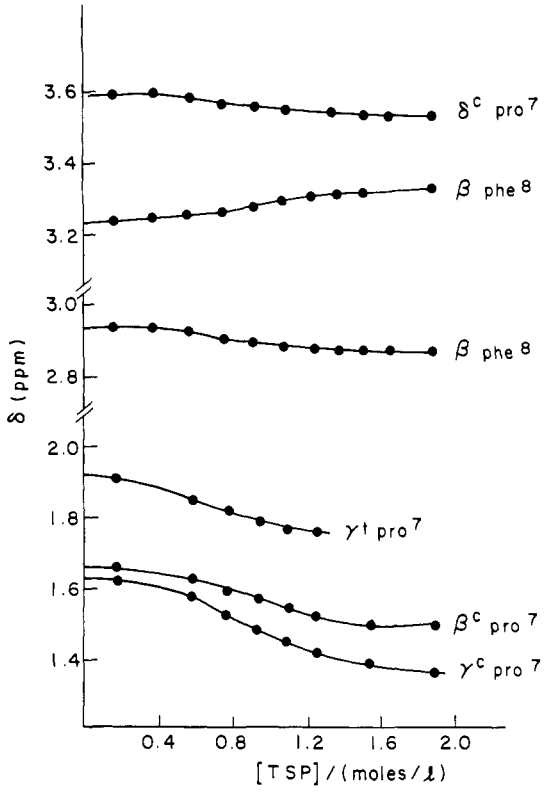

FIGURE 5: Plot of chemical shift of selected resonances of bradykinin vs. the concentration of TSP [tetradeuterio(trimethylsilyl)propionate].

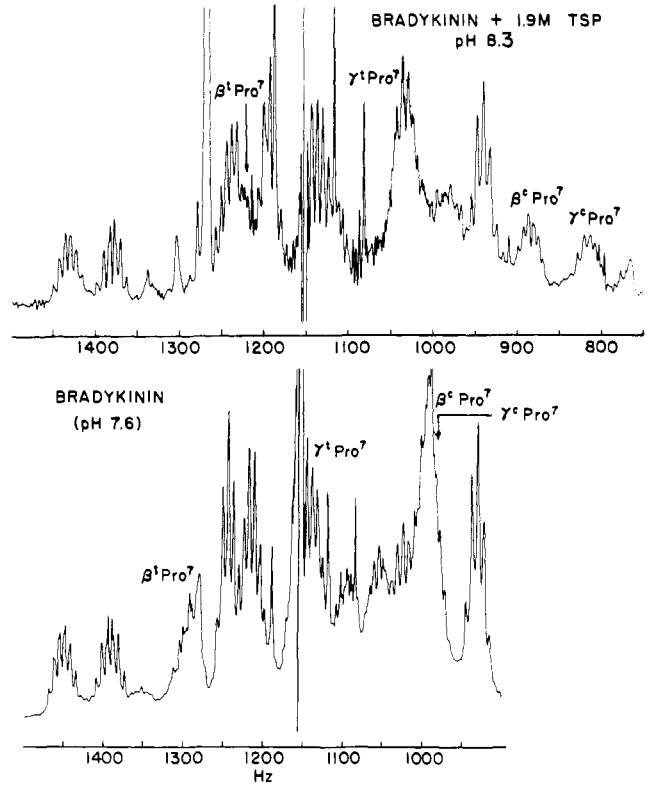

FIGURE 6: Spectra of bradykinin in aqueous solution with and without added TSP.

and δ_{BT} represent the observed chemical shift and the shifts in free BK and the complex. However, a plot of $(\delta - \delta_B)/(\delta_{BT} - \delta)$ vs. TSP concentration showed pronounced curvature. An approximately linear plot was obtained when $(\delta - \delta_B)/(\delta_{BT} - \delta)$ was plotted against the cube of TSP concentration, suggesting interaction of BK with several molecules of TSP.

The changes in shifts of the β protons of Phe[8] were accompanied by changes in both $J_{\alpha\beta(S)}$ and $J_{\alpha\beta(R)}$, with an increase in the calculated population of the χ_{60} rotamer from 0.65 to 0.82. The shifts in the Phe[8] β-proton resonances may reflect this change. The similar form of the changes in the shifts of the Ser[6] β protons suggests the same origin for these. The general downfield shifts of the Pro[7] resonances may be attributed either to a changed environment (exclusion of solvent on binding to lipid) or to a changed ring-current contribution from Phe[8] because of the conformer population change.

TSP is well below the critical micelle concentration at these levels, and it was interesting to observe the effects of a micelle-forming lipid on the BK spectrum. Accordingly, the spectrum was examined in aqueous solution at various added sodium dodecyl sulfate ($NaDodSO_4$) concentrations. The spectra at high $NaDodSO_4$ concentrations were broadened, but it could be seen that the same changes in chemical shift occurred as had been observed with TSP (Figure 6). Changes in the CD spectrum of BK in the presence of 0.1% $NaDodSO_4$ have been observed by Marlborough et al. (1976). The changes parallel those caused by transfer to trifluoroethanol as a solvent and hence indicate a hydrophobic interaction.

Discussion

The combined evidence indicates strongly that BK in aqueous solution is flexible and is in rapid equilibrium between multiple conformations, with no substantial contribution of structural features such as β turns, internal hydrogen bonds, or hydrophobic interactions. There appears to be a slight deviation in the side-chain rotameric population of Ser[6] and Phe[5] from those expected in a random coil, and an unexpected chemical shift nonequivalence of the Arg[1] δ protons suggests that the chain terminus may be interacting with a segment of the main chain. The prolines exhibit normal puckering and no preference for Ramachandran A or B forms.

BK binds weakly to TSP and $NaDodSO_4$ micelles. In both cases, BK is in fast exchange with the complex, which probably contains several molecules of lipid. Interaction occurs principally with Phe[5], Ser[6], Pro[7], and Phe[8]. The residues Phe[5], Pro[7], and Phe[8] are vitally important for the physiological activity of BK (Schroder & Hempel, 1964; Fisher et al., 1977a,b). This suggests that the mechanism of action of BK involves the interaction of these residues with a membrane or other hydrophobic surface.

Since BK exerts a variety of physiological activities at different locations and with different receptors, the ability to assume a variety of conformations may be a positive advantage.

References

Bach, A. C., II, Bothner-By, A. A., & Gierasch, L. M. (1982) *J. Am. Chem. Soc. 104*, 572–576.

Bundi, A., & Wuethrich, K. (1979) *Biopolymers 18*, 285–297.

Cann, J. R. (1972) *Biochemistry 11*, 2654–2659.

Cann, J. R., Stewart, J. M., & Matsueda, G. R. (1973) *Biochemistry 12*, 3780–3788.

Cann, J. R., Stewart, J. M., London, R. E., & Matwiyoff, N. (1976) *Biochemistry 15*, 498–504.

Cann, J. R., London, R. E., Stewart, J. M., & Matwiyoff, N. A. (1979) *Int. J. Pept. Protein Res. 14*, 388–392.

Castellano, S. M., & Bothner-By, A. A. (1964) *J. Chem. Phys. 41*, 3863–3869.

Chou, P. Y., & Fasman, G. D. (1979) *Biophys. J. 26*, 367–383.

Dadok, J., von Dreele, P. H., & Scheraga, H. A. (1972) *J. Chem. Soc., Chem. Commun.*, 1055–1056.

Feeney, J. (1976) *J. Magn. Reson. 21*, 473-478.
Fisher, G. H., Ryan, J. W., & Berryer, P. (1977a) *Cardiovasc. Med. 2*, 1179-1181.
Fisher, G. H., Chung, A., & Ryan, J. W. (1977b) *Circulation 55 and 56 (Suppl. III)*, 241.
Fisher, G. H., Marlborough, D. I., Ryan, J. W., & Felix, A. M. (1978) *Arch. Biochem. Biophys. 189*, 81-85.
Kobayashi, J., & Nagai, U. (1978) *Biopolymers 17*, 2265-2277.
Kozlowski, H., Formicka-Kozlowska, G., & Jezowska-Trzebiatowska, B. (1977) *Org. Magn. Reson. 10*, 146-150.
Lintner, K., & Fermandjian, S. (1979) *Biochem. Biophys. Res. Commun. 91*, 803-811.
Lintner, K., Fermandjian, S., Regoli, D., & Barabe, J. (1977) *Eur. J. Biochem. 81*, 395-401.
Lintner, K., Fermandjian, S., & Regoli, D. (1979) *Biochimie 61*, 87-92.
London, R. E. (1979) *Int. J. Pept. Protein Res. 14*, 377-387.
London, R. E., Stewart, J. M., Cann, J. R., & Matwiyoff, N. A. (1978) *Biochemistry 17*, 2270-2283.
Marlborough, D. I., Ryan, J. W., & Felix, A. M. (1976) *Adv. Exp. Med. Biol. 70*, 43-51.
Molday, R. S., Englander, S. W., & Kallen, R. G. (1972) *Biochemistry 11*, 150-158.
Pachler, K. G. R. (1964) *Spectrochim. Acta 20*, 581-587.
Paiva, A. C. M., & Juliano, L. (1977) *Pept., Proc. Am. Pept. Symp., 5th*, 337-339.
Philson, S. B., & Bothner-By, A. A. (1979) *Pept., Proc. Am. Pept. Symp., 6th*, 209-212.
Ptak, M., Heitz, A., & Dreux, M. (1978) *Biopolymers 17*, 1129-1148.
Schroder, E., & Hempel, R. (1964) *Experientia 15*, 529-592.
Wuethrich, K. (1976) *NMR in Biological Research: Peptides and Proteins*, Elsevier, Amsterdam.

Stabilization of Protein Structure by Sugars†

Tsutomu Arakawa and Serge N. Timasheff*

ABSTRACT: The preferential interaction of proteins with solvent components was measured in aqueous lactose and glucose systems by using a high precision densimeter. In all cases, the protein was preferentially hydrated; i.e., addition of these sugars to an aqueous solution of the protein resulted in an unfavorable free-energy change. This effect was shown to increase with an increase in protein surface area, explaining the protein stabilizing action of these sugars and their enhancing effect of protein associations. Correlation of the preferential interaction parameter with the effect of the sugars on the surface tension of water, i.e., their positive surface tension increment, has led to the conclusion that the surface free energy perturbation by sugars plays a predominant role in their preferential interaction with proteins. Other contributing factors are the exclusion volume of the sugars and the chemical nature of the protein surface.

Polyhydric alcohols and sugars have been used for many years as stabilizing agents for the maintenance of the biological activity of macromolecules (Tanford et al., 1962; Utter et al., 1964; Gerlsma, 1968, 1970; Gerlsma & Sturr, 1972, 1974; Neucere & St. Angelo, 1972; Frigon & Lee, 1972). On the other hand, cautionary notes have been sounded about the use of sucrose at high concentration, as in sucrose density gradient centrifugation, on the basis of reports that sucrose decreased the activity of some enzymes (Hinton et al., 1969). Such an alteration of enzyme activity has been ascribed usually to conformational changes without, however, any experimental evidence. Gerlsma (1968, 1970) and Gerlsma & Sturr (1972, 1974) have shown that polyhydric alcohols and sugars increased the transition temperature of some proteins in aqueous solution, and they ascribed the stabilizing action of these substances to their induction in water of a decrease in hydrogen bond rupturing potency (Gerlsma, 1970).

Another source of structural stabilization can be the preferential interaction of protein with solvent components at high concentration of additives. For example, conformational changes induced by 2-chloroethanol and guanidine hydrochloride are linked to the binding of these substances to the protein (Timasheff & Inoue, 1968; Lee & Timasheff, 1974), while protein preferential hydration is observed in aqueous solutions of 2-methyl-2,4-pentanediol (Pittz & Timasheff, 1978), which has been used successfully to crystallize ribonuclease A in its native form (King et al., 1956). The same is true of other systems, such as glycerol (Timasheff et al., 1976; Gekko & Timasheff, 1981), some salts (Timasheff et al., 1976; Arakawa & Timasheff, 1982; Aune & Timasheff, 1970), and sucrose (Lee et al., 1975), which are known to be protein structure stabilizing agents. Lee & Timasheff (1981) have analyzed thermodynamically the aqueous sucrose system and shown that the stabilizing effect of sucrose stems from the preferential hydration of proteins in this medium and that this, in turn, may be related to the increase in the free energy of cavity formation induced by addition of sucrose to water. It seemed of interest, therefore, to examine whether preferential hydration is a common feature of sugar systems and, if so, to probe the causes of such preferential hydration. A study was carried out, therefore, of interactions of solvent components with proteins in aqueous lactose and glucose solutions, and the results are presented in this paper.

Materials and Methods

All the proteins used in this study were purchased from Sigma: ribonuclease A (RNase A)[1] (type II-A, lot 87C-0207), lysozyme (lot 57C-8025), chymotrypsinogen A (lot 66C-8125), β-lactoglobulin (lot 86c-8065, 106C-8070), bovine serum al-

† From the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02254. *Received June 18, 1982.* This is Publication No. 1426. This work was supported by Grants CA 16707 and GM 14603 from the National Institutes of Health.

[1] Abbreviations: RNase A, ribonuclease A; CTG, chymotrypsinogen A; β-LG, β-lactoglobulin; BSA, bovine serum albumin; MPD, 2-methyl-2,4-pentanediol.

0006-2960/82/0421-6536$01.25/0