Amino Acid Flexibility

A Conformational Flexibility Scale for Amino Acids in Peptides**

*Fang Huang and Werner M. Nau**

The molecular flexibility of proteins is a crucial factor in determining their biological activity,[1] including binding affinity,[2] antigenicity,[3] and enzymatic activity.[4] The identification of regions in proteins with the highest conformational flexibility and rigidity is essential for predicting the mechanism of protein folding,[5,6] for understanding domain motions in proteins,[1,7,8] and for predicting the rate of nonlocal interactions as well as intramolecular reactions, including electron and proton transfer[9] and cyclizations.[10] Consequently, there is considerable interest in predicting the flexibility or, conversely, the rigidity of peptides from their amino acid sequence.[11–13] The prediction of peptide flexibility has additional implications for the de novo design of peptides[14] and for the theoretical understanding of peptide dynamics.[15]

We now report a novel scale for the flexibility of amino acids, which provides an absolute measure for the time scale of conformational changes in short structureless peptides as a function of the amino acid type. This experimental scale is derived from kinetic measurements of the collision frequency between the two ends of short random-coil polypeptides.

The present experiments provide the first application of our recently introduced fluorazophore method for measuring the kinetics of end-to-end collision in polypeptides.[16,17] In essence, 2,3-diazabicyclo[2.2.2]oct-2-ene (DBO) is attached as a fluorescent probe at one end of the peptide and tryptophan is placed at the other end as an efficient quencher (Figure 1). The unique features of this probe/quencher pair are the exceedingly long fluorescence lifetime of the probe (several hundred nanoseconds) and the contact quenching mechanism, which differentiates it from conventional fluorescence resonance energy transfer (FRET) donor/acceptor pairs. The fluorescence lifetimes of DBO/Trp peptides provide the quenching rate constants (k_q), which measure the end-to-end collision frequency (k_{coll} in Figure 1).[17] Such collisions occur on the ns–μs time scale according to recent studies.[17–20]

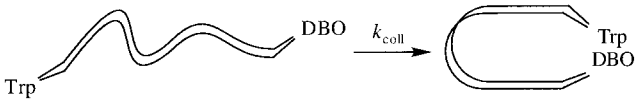
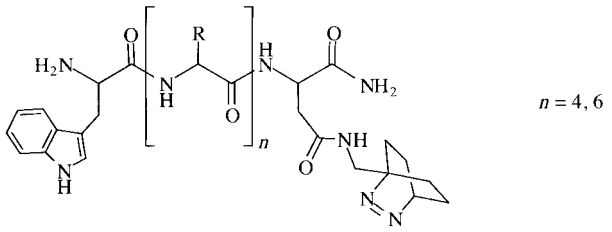


Figure 1. End-to-end collision in peptides labeled with DBO as a fluorescent probe at the *C* terminus and Trp as a fluorescence quencher at the N terminus.

We reasoned that the collision frequency of peptides, in which Trp and DBO are separated by a sequence of identical amino acids (Scheme 1), should be an excellent measure of the conformational flexibility of the backbone.[21] These peptides adopt random-coil conformations according to CD measurements.[22] Since the conformational changes required for end-to-end contact derive from fast rotations about the N–C^{α} and C^{α}–C bonds,[23] and since different α substituents are expected to hinder these bond rotations to a different degree, we expected different collision frequencies when the probe and the quencher are separated by different amino acids. This arrangement should allow us to correlate the collision frequency with the type of amino acid and thereby build up a flexibility scale (Table 1). It should be stated that this scale reflects the global or conformational flexibility of the amino acids in peptides. This scale is unrelated to previously introduced local or positional flexibility scales[24] obtained from X-ray diffraction crystallographic data.[11–13] The latter scales provide measures of the average displacements of atoms in amino acid residues from their equilibrium geometry, which are derived from vibrations on the femtosecond time scale and reflect the shallowness of the potential centered around an energy minimum.



Scheme 1. General structure of the peptides.

The quenching rate constants in Table 1, which were obtained from the fluorescence decays (Figure 2), show variations of 1–2 orders of magnitude for the various amino acids. Lower quenching rate constants correspond to lower end-to-end collision frequencies and indicate a lower flexibility (or higher rigidity). The following order of flexibility applies:

Gly > Ser > Asp, Asn, Ala > Thr, Leu > Phe, Glu, Gln > His, Arg > Lys > Val > Ile > Pro

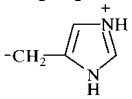
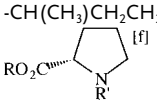
The above order was also preserved in the presence of 6 M guanidinium chloride, except that Leu appeared to give rise to a somewhat less flexible peptide. Guanidinium chloride is an efficient denaturant, which should destroy any remaining secondary structure as well as aggregation between hydro-

[*] Prof. Dr. W. M. Nau
School of Engineering and Science
International University Bremen
Campus Ring 1, 28759 Bremen (Germany)
Fax: (+49) 421-200-3229
E-mail: w.nau@iu-bremen.de
Prof. Dr. W. M. Nau, Dipl.-Chem. F. Huang
Departement Chemie
Universität Basel
Klingelbergstrasse 80, 4056 Basel (Switzerland)

[**] This work was supported by the Swiss National Science Foundation within the NFP 47 "Supramolecular Functional Materials".

 DOI: 10.1002/ange.200250684

Table 1: Fluorescence quenching rate constants in Trp-X_6-DBO-NH_2 peptides.

X	Residue (R)	k_q [10^6 s^{-1}] without additive[a]	with denaturant[b]
Gly	-H	39	23
Ser	$-CH_2OH$	25	19
Asp[c]	$-CH_2CO_2H$	21	
	$-CH_2CO_2^-$	19	12
Asn	$-CH_2CONH_2$	20	14
Ala	$-CH_3$	ca. 18[d]	ca. 14[d]
Thr	$-CH(OH)CH_3$	11	9.3
Leu	$-CH_2CH(CH_3)_2$	ca. 10[d]	ca. 5.1[d]
Phe	$-CH_2Ph$	ca. 7.6[d,e]	ca. 7.5[d]
Glu[c]	$-CH_2CH_2CO_2H$	8.8	
	$-CH_2CH_2CO_2^-$	7.4	5.4
Gln	$-CH_2CH_2CONH_2$	7.2	4.8
His[c]		4.8	4.0
Arg	$-(CH_2)_3NHCNH_2NH_2^+$	4.6	2.6
Lys[c]	$-(CH_2)_4-NH_2$	4.0	
	$-(CH_2)_4-NH_3^+$	2.8	1.9
Val	$-CH(CH_3)_2$	ca. 3.0[d,e]	ca. 2.6[d]
Ile	$-CH(CH_3)CH_2CH_3$	ca. 2.3[d,e]	ca. 1.6[d]
Pro		< 0.1	< 0.1

[a] Measured in D_2O. [b] Measured in H_2O with 6 M guanidinium chloride. [c] The unprotonated forms were studied at pD 12, the protonated ones at pD 2. [d] Extrapolated from the kinetics of the shorter peptide ($n = 4$), see Experimental Section. [e] Measured with 30% acetonitrile. [f] Entire amino acid.

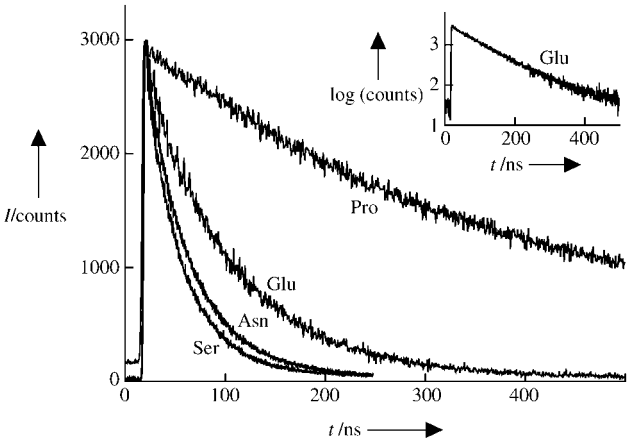


Figure 2. Fluorescence decays for selected Trp-X_6-DBO-NH_2 peptides in aerated D_2O measured by single photon counting. The inset shows a representative decay on a semi-logarithmic scale.

phobic peptides.[25] The consistency of the results in the absence and presence of this denaturant provides strong evidence that the property which is being reflected by the kinetic measurements is indeed conformational flexibility. Note also that the rate of end-to-end collision in the presence of the salt is reduced throughout the entire series of peptides; this reflects the increased viscosity of the solution which limits the mutual diffusion of the chain ends.

According to the conformational flexibility scale, the introduction of Gly into the peptide increases the conformational flexibility, while Pro makes it more rigid. This is in line with expectations from conformational space,[26] with Gly being the amino acid with the least limited rotational freedom and Pro being the one with a frozen N–C^α bond. The sequence found for the other amino acids cannot be predicted in a straightforward manner. There is a general trend between the residue size and the kinetics: larger residues lead to lower collision frequencies, which indicates a lower flexibility; for example, Gln < Asn; Thr < Ser; Phe, Leu < Ala < Gly; Ile < Val. This result is presumably related to the larger rotational barriers imposed by the larger residues and the decrease in the diffusion coefficient of the peptide chain with increasing residue size. The effect of residue charges was also examined; there appears to be a tendency for the neutral residues to allow a slightly greater flexibility, namely, unprotonated Lys > protonated Lys, protonated Glu > unprotonated Glu, and protonated Asp ≧ unprotonated Asp. The observed charge effect is not very large, but is in line with the intuitive expectation that Coulombic repulsions reduce the overall flexibility by limiting the conformational freedom.

β Branching is well known to increase the activation barriers for bond rotations of an amino acid and therefore decreases the accessible conformational space in a peptide significantly.[27] Indeed, the peptides with β-substituted amino acids (Val and Ile) are amongst the least flexible ones on our scale. Particularly instructive is the comparison of the two constitutional isomers Leu and Ile, where the latter one imposes a higher rigidity. The β-branched Thr gives rise to more flexible peptides than Val and Ile. Nevertheless, the flexibility of the Thr peptides is slightly decreased compared to those composed of β-unsubstituted amino acids with even larger residues, for example, Thr < Asn, Asp. Evidently, the β-hydroxy group somewhat limits the flexibility, but much less severely than the β-methyl group in Val and Ile. The special effect of hydroxy substitution is also evident with Ser, which gives rise to a more flexible peptide than Ala, although it is larger as a result of the additional hydroxy group.

We did not observe a clear-cut relationship between the flexibility and the hydrophobicity of the residue. Val and Ile are very hydrophobic amino acids, but the high rigidity of the related peptides appears to be mainly a consequence of the β-substitution pattern, since there is a large differentiation from Leu, which is similarly hydrophobic. On the other hand, His and Arg are quite hydrophilic, but their peptides are less flexible than that based on the hydrophobic Phe. The hydrophobicity of the residues, therefore, does not emerge as a common denominator of peptide flexibility.

A long-standing question in peptide chemistry is which properties of an amino acid are decisive for the protein secondary structure. Statistical,[28–30] theoretical,[31] and experimental[32,33] analyses have revealed relative abundances of particular amino acids in secondary protein structures such as α helices, β strands, and β turns. According to the statistical results,[28] Met, Glu, Leu, Ala, Gln, Lys, His, and Cys are α-helix-forming amino acids, Val, Ile, Phe, Tyr, Thr, and Trp tend to form β-sheet structures, and Pro, Gly, Asp, Ser, and Asn favor β turns. A correlation between conformational

flexibility and the propensity of an amino acid to occur in a secondary structure emerges if one disregards the α-helix-forming amino acids and Pro (see below). Accordingly, the most flexible amino acids, including Gly, Ser, Asn, and Asp, are β-turn-forming amino acids, while the most rigid amino acids, namely Val and Ile, are β-sheet-forming amino acids. In addition, when comparing the β-branched amino acids, Thr is not only the most flexible one on our scale, but it also has a much higher tendency than Val and Ile to occur in β turns.[28]

We consider that this agreement between the flexibility and the β-turn and β-sheet propensity is significant and suggest that both properties are the result of the same underlying reason: namely, the (energetically unfavorable) angular arrangements which are required in β turns may be the same as those which need to be reached for collision-induced quenching (Figure 1). In other words, the conformations required for quenching are turnlike. Large α substituents and in particular β branching do not favor β turns because of steric interactions,[27] and this avoidance also manifests itself in the kinetic measurements where a similar conformation is required for quenching. It appears that flexible amino acids, in particular Gly and Ser, promote stable β turns, although one must keep in mind that the abundance of an amino acid also depends strongly on the specific position within a β turn.[34] Rigid amino acids have a higher tendency to occur in β-sheet structures than in β turns, but they may also favor the formation of α helices. α-Helix formation is dominated by additional interactions between non-adjacent amino acids,[35] which are unlikely to be related to chain flexibility. In fact, a relationship between α-helix propensity and flexibility cannot be recognized, although it is evident that the α-helix-forming amino acids adopt an intermediate position on the proposed flexibility scale.

A clear exception is Pro,[22] which gives rise to the most rigid peptide, but which is also favored in β turns. The high rigidity is imposed by its cyclic structure, which prevents rotations about the N–C^{α} bond. The high β-turn propensity, on the other hand, is related to the restricted $-60°$ ϕ angle, which presents an ideal arrangement in the $i+1$ position of β turns.[36]

In summary, the following trends emerge for the conformational flexibility of amino acids in peptides as assessed by our method: 1) Large residues reduce the flexibility; 2) β-alkyl branching greatly reduces flexibility, while β-hydroxy substitution retains a high flexibility; 3) charge repulsions between residues decrease the flexibility slightly; 4) the conformationally frozen amino acid Pro is the least flexible while the simplest amino acid Gly is the most flexible; 5) a correlation with β-turn propensity applies, with the most flexible amino acids also being those with high β-turn propensity; 6) rigid amino acids appear to be favored in β sheets; and 7) there is no clear-cut relationship between flexibility and hydrophobicity or the α-helix propensity of amino acids. Finally, one must note that the present flexibility scale is based on a set of synthetic peptides with a backbone composed of identical amino acids. Whether the flexibility of native random-coil peptide sequences can be predicted from this scale in an incremental fashion or depends strongly on the specific sequence, will be the subject of future studies applying the same methodology.

Experimental Section

The peptides were commercially synthesized in >95% purity (Affina, Berlin). Details on the synthesis of the probe and its suitability in solid-phase peptide synthesis have been reported.[17] The choice of the peptide length (number of amino acids as spacers of the probe and the quencher) is essential since the peptide should not be too long to enable measurement of significant quenching and to maintain water solubility, and neither should it be too short to maintain sufficiently long lifetimes for accurate measurement and to ensure that the measured rates reflect an intrinsic property of the backbone, namely, the amino acid. Six amino acids ($n=6$) provided a good balance between these factors, but peptides of this chain length with the most hydrophobic amino acids (Ala, Leu, Phe, Val, and Ile) were poorly soluble in pure water, thus causing complications in both peptide synthesis as well as fluorescence measurement. For these cases, a shorter sequence of four amino acids ($n=4$) was chosen, and the known length dependence of the kinetics of end-to-end collision[17] allowed extrapolation of the rates to the value for $n=6$.

Fluorescence decays were measured by single photon counting (FLS900, Edinburgh Instruments) under air, either in D_2O or in H_2O containing 6 M guanidinium chloride. All peptides were sufficiently soluble in the presence of guanidinium chloride, but peptides based on the hydrophobic amino acids Phe, Val, and Ile were not sufficiently soluble without this denaturant. In these three cases, 30% acetonitrile was added to D_2O to achieve the same working concentration (10 μM) as for the other peptides and these solutions were deaerated. The co-solvent did not have a major effect on the measured rates, which could be demonstrated through control experiments in neat D_2O at lower peptide concentration (ca. 1 μM).

The decay kinetics were monoexponential in all cases (Figure 2), such that it was possible to assign a characteristic fluorescence lifetime (τ) to each peptide and amino acid backbone.[21] The quenching rate constants k_q were obtained from the experimental fluorescence lifetimes as $k_q = 1/\tau - 1/\tau_0$, where τ_0 corresponds to the intrinsic fluorescence lifetime of the probe in the absence of quenchers, for example, as measured for the free parent fluorophore as well as for peptides lacking a quencher. This value amounts to 500 ns in aerated D_2O and to 360 ns in H_2O containing 6 M guanidinium chloride. Subject to the assumption of a random coil conformation[22] and subject to the assumption of diffusion-controlled intramolecular quenching,[17,20] the quenching rate constant (k_q) can be directly interpreted as end-to-end collision frequency (k_{coll} in Figure 1).

Received: December 3, 2002 [Z50684]

Keywords: amino acids · fluorescence · peptides · protein folding · secondary structure

[1] R. Huber, *Biochem. Soc. Trans.* **1987**, *15*, 1009–1020.
[2] D. T. Braddock, J. M. Louis, J. L. Baber, D. Levens, G. M. Clore, *Nature* **2002**, *415*, 1051–1056.
[3] N. Taschner, S. A. Müller, V. R. Alumella, K. N. Goldie, A. F. Drake, U. Aebi, T. Arvinte, *J. Mol. Biol.* **2001**, *310*, 169–179.
[4] P. Závodszky, J. Kardos, A. Svingor, G. A. Petsko, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 7406–7411.
[5] P. Hammarström, U. Carlsson, *Biochem. Biophys. Res. Commun.* **2000**, *276*, 393–398.
[6] A. R. Fersht, *Curr. Opin. Struct. Biol.* **1997**, *7*, 3–9.
[7] Y. Q. Qian, G. Otting, K. Furukubo-Tokunaga, M. Affolter, W. J. Gehring, K. Wüthrich, *Proc. Natl. Acad. Sci. USA* **1992**, *89*, 10738–10742.
[8] S. Hayward, *Proteins* **1999**, *36*, 425–435.
[9] D. Pogocki, E. Ghezzo-Schöneich, C. Schöneich, *J. Phys. Chem. B* **2001**, *105*, 1250–1259.

[10] M. Mutter, *J. Am. Chem. Soc.* **1977**, *99*, 8307 – 8314.
[11] P. A. Karplus, G. E. Schulz, *Naturwissenschaften* **1985**, *72*, 212 – 213.
[12] R. Bhaskaran, P. K. Ponnuswamy, *Int. J. Pept. Protein Res.* **1988**, *32*, 241 – 255.
[13] M. Vihinen, E. Torkkila, P. Riikonen, *Proteins* **1994**, *19*, 141 – 149.
[14] S. H. Gellman, *Acc. Chem. Res.* **1998**, *31*, 173 – 180.
[15] W. F. van Gunsteren, R. Bürgi, C. Peter, X. Daura, *Angew. Chem.* **2001**, *113*, 363 – 367; *Angew. Chem. Int. Ed.* **2001**, *40*, 351 – 355.
[16] W. M. Nau, X. Wang, *ChemPhysChem* **2002**, *3*, 393 – 398.
[17] R. R. Hudgins, F. Huang, G. Gramlich, W. M. Nau, *J. Am. Chem. Soc.* **2002**, *124*, 556 – 564.
[18] O. Bieri, J. Wirz, B. Hellrung, M. Schutkowski, M. Drewello, T. Kiefhaber, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 9597 – 9601.
[19] L. J. Lapidus, P. J. Steinbach, W. A. Eaton, A. Szabo, J. Hofrichter, *J. Phys. Chem. B* **2002**, *106*, 11628 – 11640.
[20] I.-C. Yeh, G. Hummer, *J. Am. Chem. Soc.* **2002**, *124*, 6563 – 6568.
[21] Most amino acids have been shown not to act as quenchers. Only Trp, Tyr, Cys, and Met are themselves quenchers (ref. [17]) and these four amino acids could consequently not be included in the present study; the fluorescence lifetimes of peptides containing these amino acids in their backbone would be too short. Also His becomes a fairly strong quencher in its unprotonated form (above pH 6, ref. [17]), such that His could only be studied at low pH values in its protonated form.
[22] CD measurements were performed for all peptides (including measurements at different pH values, for example, for protonated and unprotonated Lys and Glu). The CD patterns are characteristic of random-coil conformations, which excludes the occurrence of sizable amounts of secondary structures in any of the investigated short peptides, including the peptides composed of amino acids with known α-helix propensity. The only exception was the Pro-derived peptide since, according to the CD spectrum, it adopts its characteristic PPII helix.
[23] D. V. Mikhailov, L. Washington, A. M. Voloshin, V. A. Daragan, K. H. Mayo, *Biopolymers* **1999**, *49*, 373 – 383.
[24] J. Koca, *Prog. Biophys. Mol. Biol.* **1998**, *70*, 137 – 173.
[25] D. Voet, J. G. Voet, C. W. Pratt, *Fundamentals of Biochemistry*, J. Wiley and Sons, New York, **1999**.
[26] P. Chakrabarti, D. Pal, *Prog. Biophys. Mol. Biol.* **2001**, *76*, 1 – 102.
[27] D. Pal, P. Chakrabarti, *Acta Crystallogr. D* **2000**, *56*, 589 – 594.
[28] M. Levitt, *Biochemistry* **1978**, *17*, 4277 – 4285.
[29] E. G. Hutchinson, J. M. Thornton, *Protein Sci.* **1994**, *3*, 2207 – 2216.
[30] V. Muñoz, L. Serrano, *Proteins* **1994**, *20*, 301 – 311.
[31] P. Koehl, M. Levitt, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 12524 – 12529.
[32] C. N. Pace, J. M. Scholtz, *Biophys. J.* **1998**, *75*, 422 – 427.
[33] M. Monné, M. Hermansson, G. von Heijne, *J. Mol. Biol.* **1999**, *288*, 141 – 145.
[34] P. Y. Chou, G. D. Fasman, *Biophys. J.* **1979**, *26*, 367 – 383.
[35] A. G. Street, S. L. Mayo, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 9074 – 9076.
[36] A. Perczel, I. Jákli, B. M. Foxman, G. D. Fasman, *Biopolymers* **1996**, *38*, 723 – 732.

