Article

Effect of Thioxopeptide Bonds on #-Helix Structure and Stability

Andreas Reiner, Dirk Wildemann, Gunter Fischer, and Thomas Kiefhaber

J. Am. Chem. Soc., **2008**, 130 (25), 8079-8084 • DOI: 10.1021/ja8015044 • Publication Date (Web): 31 May 2008

More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Links to the 1 articles that cite this article, as of the time of this article download
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

View the Full Text HTML

J|A|C|S
ARTICLES

Published on Web 05/31/2008

Effect of Thioxopeptide Bonds on α-Helix Structure and Stability

Andreas Reiner,[†] Dirk Wildemann,[‡] Gunter Fischer,[‡] and Thomas Kiefhaber[†,*]

Chemistry Department, Institute for Biophysical Chemistry, Technische Universität München and Munich Center for Integrated Protein Science, Lichtenbergstrasse 4, D-85747 Garching, Germany and the Max Planck Research Unit for Enzymology of Protein Folding, Weinbergweg 22, D-06120 Halle (Saale), Germany

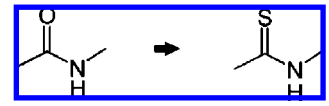
Received February 28, 2008; E-mail: t.kiefhaber@tum.de

Abstract: Thioxoamide (thioamide) bonds are nearly isosteric substitutions for amides but have altered hydrogen-bonding and photophysical properties. They are thus well-suited backbone modifications for physicochemical studies on peptides and proteins. The effect of thioxoamides on protein structure and stability has not been subject to detailed experimental investigations up to date. We used alanine-based model peptides to test the influence of single thioxoamide bonds on α-helix structure and stability. The results from circular dichroism measurements show that thioxoamides are strongly helix-destabilizing. The effect of an oxo-to-thioxoamide backbone substitution is of similar magnitude as an alanine-to-glycine substitution resulting in a helix destabilization of about 7 kJ/mol. NMR characterization of a helical peptide with a thioxopeptide bond near the N-terminus indicates that the thioxopeptide moiety is tolerated in helical structures. The thioxoamide group is engaged in an *i*, *i*+4 hydrogen bond, arguing against the formation of a 3_{10}-helical structure as suggested for the N-termini of α-helices in general and for thioxopeptides in particular.

Introduction

Proteins are built from a small set of amino acids linked by peptide bonds. They can form systems of high complexity and versatility despite the simplicity of their building blocks. The introduction of non-natural side chains or backbone linkages allows the study, alteration, or expansion of the basic properties of polypeptide chains. The polypeptide backbone plays an important role in protein structure, stability, and dynamics, since it mediates solvation,[1,2] restricts conformational space,[3] and forms hydrogen bonds. Drastic changes in the backbone properties as introduced by glycine and proline residues strongly alter the structural and dynamic properties of polypeptide chains.[4,5] Numerous synthetic backbone modifications have been introduced, encompassing β-peptides, peptides with reversed stereochemistry or direction, and several kinds of amide substitutions, e.g., exchanges to tertiary amides, esters, aldehydes, olefins, or thioesters. The replacement of the amide bond by a thioxoamide (thioamide) bond (Ψ[CS—NH]) represents a conservative modification of the peptide backbone (Scheme 1), which has been envisaged to improve the bioactivity and stability of peptidomimetica (for examples, see refs 6 and 9). Thioxoamide groups are nearly but not completely isosteric to amides. The C═S bond is 37% longer than the C═O bond (1.65 Å vs 1.20 Å), and sulfur has a 32% larger van der Waals radius than oxygen (1.85 Å vs 1.40 Å). Theoretical studies indicate that the conformational space of the residues preceding and following a thioxoamide is more restricted compared to an amide bond.[10–13] Although the energetic estimates differ, it was predicted that the typical ϕ,ψ-regions for the peptide backbone should remain accessible, including those corresponding to β-sheets, β-turns, and right-handed α-helices. Up to date a single

Scheme 1. Oxo-to-Thioxoamide Substitution in a Peptide Bond



[†] Technische Universität München.
[‡] Max Planck Research Unit for Enzymology of Protein Folding.

(1) Baldwin, R. L. *Biophys. Chem* **2002**, *101* (102), 203–210.
(2) Baldwin, R. L. Weak interactions in protein folding: hydrophobic free energy, van der Waals interactions, peptide hydrogen bonds and peptide solvation. In *Protein Folding Handbook*; Buchner, J., Kiefhaber,T., Eds.; Wiley/VCH: Weinheim, 2005; Vol. *1*, pp 127−162..
(3) Ramachandran, G. N.; Sasisekharan, V. *Adv. Protein Chem.* **1968**, *23*, 283–437.
(4) Flory, P. J. Statistical Mechanics of Chain Molecules; Hanser Publishers: Munich, 1969.
(5) Krieger, F.; Möglich, A.; Kiefhaber, T. *J. Am. Chem. Soc.* **2005**, *127*, 3346–3352.
(6) Seebach, D.; Ko, S. Y.; Kessler, H.; Köck, M.; Reggelin, M.; Schmieder, P.; Walkinshaw, M. D.; Bölsterli, J. J.; Bevec, D. *Helv. Chim. Acta* **1991**, *74*, 1953–1990.
(7) Kessler, H.; Matter, H.; Geyer, A.; Diehl, H.-J.; Köck, M.; Kurz, G.; Opperdoes, F. R.; Callens, M.; Wierenga, R. K. *Angew. Chem., Int. Ed. Engl.* **1992**, *31*, 328–330.
(8) Yao, S.; Zutshi, R.; Chmielewski, J. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 699–704.
(9) Frank, R.; Jakob, M.; Thunecke, F.; Fischer, G.; Schutkowski, M. *Angew. Chem., Int. Ed.* **2000**, *39*, 1120–1122.
(10) Balaji, V. N.; Profeta, S.; Dietrich, S. W. *Biochem. Biophys. Res. Commun.* **1987**, *145*, 834–841.
(11) Cour, T. F. M. *Int. J. Peptide Protein Res.* **1987**, *30*, 564–571.
(12) Artis, D. R.; Lipton, M. A. *J. Am. Chem. Soc.* **1998**, *120*, 12200–12206.
(13) Tran, T. T.; Treutlein, H.; Burgess, A. W. *J. Comput. Chem.* **2001**, *22*, 1026–1037.

example of a naturally occurring protein was reported, which contains a backbone thioxopeptide bond.[14] Thioxoamides can serve as both hydrogen bond donors and acceptors. In mixed hydrogen bonds between thioxoamides and amides, the thioxoamide is predicted to be a weaker hydrogen bond acceptor and a better hydrogen bond donor compared to an amide.[15] Therefore thioxopeptide groups may be suitable to evaluate the contribution of single hydrogen bonds to the stability, folding, and function of proteins. A special feature of the thioxopeptide bond arises from the red-shifted absorption bands and the higher rotational barrier for the *cis*−*trans* isomerization.[16,17] This property allows a selective shift of the equilibrium either to the *cis* or to the *trans* isomerization state by irradiation with UV light of different wavelengths. The photoinduced isomerization is efficient and fast (<600 ps),[18–20] whereas thermal relaxation back to equilibrium is slow (>10 min).[9,16] Thioxopeptides are therefore good candidates for fast photoswitches in proteins, either to regulate biological or enzymatic activity[21] or to initiate conformational transitions for time-resolved studies.

In order to use the potential of thioxoamide substitutions to study protein structure, folding, and dynamics, it is important to evaluate their effects on protein stability. The introduction of thioxoamides at hydrogen-bonded positions in β-sheets and α-helices should be highly unfavorable, since these structures are optimally packed to accommodate the shorter amide−amide hydrogen bonds (2.1 Å for an amide−amide hydrogen bond vs 2.7 Å for a thioxoamide−amide hydrogen bond).[11] In an experimental study on a thioxoamide substituted β-hairpin peptide, steric clashes were avoided by placing the sulfur in a non-hydrogen-bonded turn position.[22] The results suggested hairpin formation in the thioxylated peptide but did not address the effect on hairpin stability. In α-helices all carbonyl groups except for those of the four C-terminal residues are involved in *i*, *i*+4 hydrogen bonds. Thioxoamides are unlikely to fit into the tight groove between two helical turns, which might cause helix disruption or kink formation.[11] It was further proposed that thioxopeptide groups favor the 3_{10}-helix conformation, since it is better suited to accommodate the larger sulfur.[23] Surprisingly, an experimental study on a dimeric α-helical coiled-coil structure (GCN4) reported that thioxopeptide groups are structurally tolerated and even a slight stabilizing effect on the coiled-coil was observed.[24] The contradiction to theoretical considerations was attributed to favorable contributions from hydrogen bonding of the thioxoamide group. However, the two positions were chosen[24] to place the larger sulfur atom in a widened part of the slightly distorted helix structure present in coiled-coils.[25]

To investigate the effect of thioxoamides on α-helix structure and stability we introduced single amide-to-thioxoamide substitutions at a central and a N-terminal position in alanine-based helical peptides. Using circular dichroism (CD) and nuclear magnetic resonance (NMR) spectroscopy we tested the effect of thioxopeptide bonds on α-helix structure and stability.

Table 1. Amino Acid Sequences of the Peptides Used in This Study

	central substitution
cΨ[a]	Ac-KAAAA KAA**Ψ**AA KAAAA K-NH_2
cA	Ac-KAAAA KAA **A**A KAAAA KGY-NH_2
cG	Ac-KAAAA KAA **G**A KAAAA KGY-NH_2
cP	Ac-KAAAA KAA **P**A KAAAA KGY-NH_2
	N-terminal substitution
nΨ	Ac-DF**Ψ**AAA KAAAA KAAAA K-NH_2
nA	Ac-DF **A**AA KAAAA KAAAA K-NH_2
nG	Ac-DF **G**AA KAAAA KAAAA K-NH_2
nP	Ac-DF **P**AA KAAAA KAAAA K-NH_2

[a] Ψ denotes the substitution of the amide bond [CO−NH] by a thioxoamide bond [CS−NH].

Results and Discussions

Thioxoamide Substitution at a Central Helix Position. We chose a well-characterized 16 amino acid alanine-based model helix[26–31] to investigate the effect of single thioxopeptide substitutions on α-helix structure and stability (Table 1). The peptides contain lysine residues at every fifth position to increase solubility and were shown to be monomeric.[26] Capping of the termini prevents unfavorable electrostatic interactions with the helix dipole. This peptide was shown to display a helix content of ∼70%.[27,30] We synthesized a peptide with the central amide bond between Ala8 and Ala9 substituted by a thioxoamide bond (Ψ[CS−NH]), indicated by the short notation **cΨ** (Table 1). The helix content is expected to reach a maximum in the central region of the peptide,[32] rendering this part especially sensitive to perturbations.[27] To characterize the effect of the thioxopeptide substitution we synthesized three reference peptides. One peptide has the same amino acid sequence as **cΨ** but contains the regular oxopeptide bond between Ala8 and Ala9 (**cA**). The other two peptides also have an oxopeptide bond between residues 8 and 9 but have Ala9 replaced by the helix-destabilizing amino acids glycine (**cG**) or proline (**cP**).

To test the effect of the thioxopeptide bond on helix structure and stability we recorded far-UV CD spectra of all peptides. Figure 1 compares the spectrum of **cΨ** to the spectra of the reference peptides. The **cΨ** peptide is significantly less structured than the **cA** peptide, which shows a characteristic α-helical

(14) Grabarse, W.; Mahlert, F.; Shima, S.; Thauer, R. K.; Ermler, U. *J. Mol. Biol.* **2000**, *303*, 329–344.
(15) Aleman, C. *J. Phys. Chem. A* **2001**, *105*, 6717–6723.
(16) Zhao, J.; Wildemann, D.; Jakob, M.; Vargas, C.; Schiene-Fischer, C. *Chem. Commun.* **2003**, 2810–2811.
(17) Wiberg, K. B.; Rush, D. J. *J. Am. Chem. Soc.* **2001**, *123*, 2038–2046.
(18) Satzger, H.; Root, C.; Gilch, P.; Zinth, W.; Wildemann, D.; Fischer, G. *J. Phys. Chem. B* **2005**, *109*, 4770–4775.
(19) Cervetto, V.; Bregy, H.; Hamm, P.; Helbing, J. *J. Phys. Chem. A* **2006**, *110*, 11473–11478.
(20) Helbing, J.; Bregy, H.; Bredenbeck, J.; Pfister, R.; Hamm, P.; Huber, R.; Wachtveitl, J.; DeVico, L.; Olivucci, M. *J. Am. Chem. Soc.* **2004**, *126*, 8823–8834.
(21) Wildemann, D.; Schiene-Fischer, C.; Aumüller, T.; Bachmann, A.; Kiefhaber, T.; Lücke, C.; Fischer, G. *J. Am. Chem. Soc.* **2007**, *129*, 4910–4918.
(22) Miwa, J. H.; Patel, A. K.; Vivatrat, N.; Popek, S. M.; Meyer, A. M. *Org. Lett.* **2001**, *3*, 3373–3375.
(23) Tran, T. T.; Zeng, J.; Treutlein, H.; Burgess, A. W. *J. Am. Chem. Soc.* **2002**, *124*, 5222–5230.
(24) Miwa, J. H.; Pallivathucal, L.; Gowda, S.; Lee, K. E. *Org. Lett.* **2002**, *4*, 4655–4657.
(25) Goodman, E. M.; Kim, P. S. *Biochemistry* **1991**, *30*, 11615–11620.
(26) Marqusee, S.; Robbins, V. H.; Baldwin, R. L. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 5286–5290.
(27) Chakrabartty, A.; Schellman, J. A.; Baldwin, R. L. *Nature* **1991**, *351*, 586–588.
(28) Scholtz, J. M.; Qian, H.; York, E. J.; Stewart, J. M.; Baldwin, R. L. *Biopolymers* **1991**, *31*, 1463–1470.
(29) Scholtz, J. M.; Baldwin, R. L. *Annu. Rev. Biophys. Biomol. Struct.* **1992**, *21*, 95–118.
(30) Chakrabartty, A.; Kortemme, T.; Baldwin, R. L. *Protein Sci.* **1994**, *3*, 843–852.
(31) Rohl, C. A.; Chakrabartty, A.; Baldwin, R. L. *Protein Sci.* **1996**, *5*, 2623–2637.
(32) Lifson, S.; Roig, A. *J. Chem. Phys.* **1961**, *34*, 1963–1974.

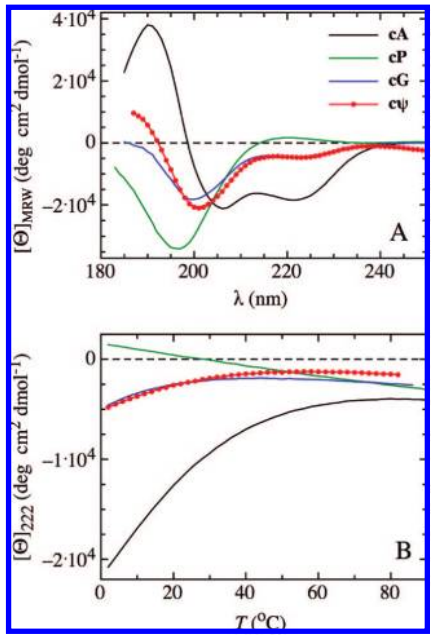


Figure 1. (A) Far-UV CD spectra of the peptides with central substitutions at 5 °C. (B) Thermal melting of the peptides monitored at 222 nm. The peptide sequences are given in Table 1. The data were recorded in 10 mM potassium phosphate, pH 7.0.

spectrum with minima at 222 and 208 nm and a maximum at 190 nm. However, **cΨ** retains some helical structure as indicated by minima around 222 and 201 nm. In the far-UV region the CD spectrum of **cΨ** is comparable to the spectrum of the peptide with the glycine substitution (**cG**), whereas the reference peptide with a proline residue (**cP**) shows a typical random coil spectrum with a maximum around 218 nm and a minimum around 197 nm. The small differences between the spectra of **cΨ** and **cG** below 215 nm are probably due to contributions from the thioxoamide bond, which has an additional absorbance band centered around 210 nm.[33] The near-UV CD spectrum of **cΨ** differs from the spectra of all reference peptides by an additional negative band around 266 nm and a very weak positive band around 350 nm (Figure S1). These bands originate from the red-shifted $\pi_o\pi^*$ transition and the $n\pi^*$ transition, respectively, of the thioxoamide group.[33]

In order to quantify the destabilizing effect of the thioxopeptide bond, we calculated the helicity of the peptides based on the mean residual ellipticity at 222 nm ($[\Theta]_{222}$) in the presence of 1 M NaCl (Table 2). The presence of NaCl was shown to allow a more reliable determination of the helical content in alanine-based model peptides and is commonly used for a quantitative analysis.[30,31] For the calculation of the helical content we used values of $[\Theta]_H = -44\,000\cdot(1-3/N)$ deg cm^2 dmol^{-1}, where N is the number of residues and $[\Theta]_C = 2220$ deg cm^2 dmol^{-1} for $[\Theta]_{222}$ of the 100% helical and 100% unfolded state, respectively.[34] At 222 nm the influence of the thioxopeptide group on the CD spectrum of the unfolded state is small, since thioxopeptide groups in unstructured alanine peptides give only a weak CD signal, which is, however, slightly larger than the CD signal of an oxopeptide bond.[33] We do not know the CD signal of a thioxoamide bond in the helical conformation, but the contribution of a single thioxoamide bond to the CD signal in the 16 amino acid peptide is expected to be small. Based on these considerations, we assume an upper limit for the error of the calculated helix content in our single thioxoamide bond containing peptides of ~8%. For **cΨ** this results in a helix content of ~24 ± 8% compared to helix contents of 68 ± 5%, 26 ± 5%, and 6 ± 5% (Table 2) for **cA**, **cG**, and **cP**, respectively. The values for the peptides with all-oxoamide bonds are in good agreement with reported values for similar peptides.[30]

Table 2. Helix Content Obtained from CD Spectroscopy and Helix Propagation Parameters Obtained from Lifson−Roig Theory

peptide	$[\Theta]_{222}$[a] (deg cm^2 dmol^{-1})	f_H[b] (%)	w[c]	$\Delta\Delta G^{\circ}$[d] (kJ mol^{-1})
cΨ	−6900	24 ± 8	0.044	7.2
cA	−24 200	68 ± 5	1.70[e]	0
cG	−7900	26 ± 5	0.050	7.0
cP	−290	6 ± 5	<0.001	>15.8
nΨ	−16 000	48 ± 8	n.d.[f]	n.d.
nA	−23 900	69 ± 5	n.d.	n.d.
nG	−17 000	51 ± 5	n.d.	n.d.
nP	−14 200	43 ± 5	n.d.	n.d.

[a] $[\Theta]_{MRW}$ at 222 nm measured in 10 mM potassium phosphate, pH 7.0 at 0 °C. For the centrally substituted peptides 1 M NaCl was added. [b] Mean helix content. The error is estimated to be <5%, for **cΨ** < ±8%. [c] Propagation parameter for the single substitutions, obtained using Lifson−Roig theory with the parameters given in ref 31. [d] Free enthalpy difference for helix formation compared to alanine at 0 °C. The values were calculated using $\Delta G^{\circ} = -RT\ \ln(w/(1+v))$. [e] Not fitted, since it is part of the parameter set used for analysis.[31] Using this value Lifson−Roig theory predicts a helix content of 69% for **cA**. [f] The N-terminal position is not very sensitive to perturbations. Thus, no attempt was made to calculate Lifson−Roig parameters from the CD signals of the N-terminally substituted peptides.

The helical content of the different peptides can further be obtained from the thermal melting behavior monitored by the change in CD at 222 nm (Figure 1B). The unfolding transitions of the **cΨ** and **cG** peptides show similar cooperativity and a similar change in ellipticity. At low temperature the $[\Theta]_{222}$-values of the two peptides and the change in $[\Theta]_{222}$ with temperature are almost identical. These findings provide further evidence for similar stability and helicity of the **cΨ** and **cG** peptides. The $[\Theta]_{222}$-value of the unfolded baseline is increased by about 1000 deg cm^2 dmol^{-1} in **cΨ** as expected from the contributions of the thioxoamide to the CD signal of the unfolded state, but shows the same linear decrease with increasing temperature as in the **cG** peptide. The **cA** peptide shows a much larger signal change upon thermal unfolding due to its higher helix content. The **cP** peptide exhibits a linear decrease in CD signal with increasing temperature, in accordance with the presence of a predominately unfolded conformation at all temperatures.[34] Temperature-induced structural changes in the **cΨ** peptide are also observed when the additional CD bands of the thioxoamide bond at 266 and 350 nm are monitored (Figure S1). However, the CD changes upon helix unfolding in this region are much smaller than those at 222 nm.

A quantitative evaluation of the effect of side chain or backbone modifications on helix stability can be obtained using Lifson−Roig theory for helix−coil transition.[32] In Lifson−Roig theory the propagation parameter, w^{Xaa}, is a statistical weight that describes the tendency of a single amino acid residue to adopt the helical conformation. It can be interpreted as an equilibrium constant between a residue in the coiled and in the helical state. A w^{Ala}-value of 1.70 has been determined for alanine, the amino acid with the highest tendency to adopt the helical conformation.[31] To evaluate the effect of a single

(33) Hollósi, M.; Kollát, E.; Kajtár, J.; Kajtár, M.; Fasman, G. D. *Biopolymers* **1990**, *30*, 1061–1072.
(34) Luo, P.; Baldwin, R. L. *Biochemistry* **1997**, *36*, 8413–8421.

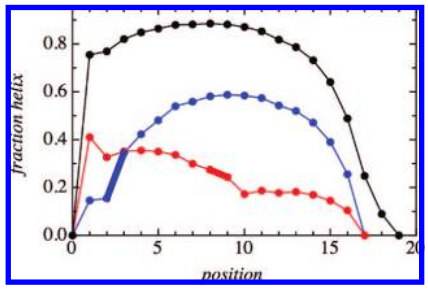


Figure 2. Position-dependent helix content of the peptides **cA** (black), **cΨ** (red), and **nΨ** (blue) as predicted by Lifson−Roig theory with the parameters given in Table 2. The bold line indicates the location of the thioxopeptide bond.

thioxoamide bond on helix stability we ascribe a special propagation parameter, w^{thio}, to the residue C-terminal of the thioxoamide bond (Table 2). To determine the w^{thio}-value, we used the w^{Xaa}-values for the standard amino acids and a weight for the boundary positions, v, of 0.036. Additional weights were introduced to account for capping effects at the helix ends.[31,35] Analysis of the **cΨ** peptide shows that the highly destabilizing effect of the thioxopeptide group is reflected in a low propagation parameter, w^{thio}, of 0.044, which corresponds to a helix destabilizing effect of ~7 kJ/mol relative to an oxopeptide bond in the **cA** reference peptide (Table 2). This leads to a major decrease of helical content in the central part of the α-helix (Figure 2). The w^{thio}-value is almost identical to the value of 0.050 determined for w^{Gly} in the **cG** peptide (Table 2). It should be noted that a thioxopeptide bond in a central helix position involves hydrogen bonds to amides four residues apart in both directions and therefore might have a less local effect than the glycine and proline substitutions in the reference peptides.

Thioxoamide Substitution in the N-Terminal Region of an α-Helix. The results on **cΨ** have shown that a thioxopeptide group in the central region of an α-helix has a strongly helix-destabilizing effect (Figure 1). The terminal regions of helical peptides exhibit less helical content compared to the central parts, and thus perturbations in this region have less effect on helix content.[27] To study the effect of a thioxopeptide bond on helix structure and stability in a less destabilized environment, we placed an oxo-to-thioxo substitution between residues 2 and 3 in an alanine-based peptide (**nΨ**; Table 1). In addition, Lys1 was replaced by a stabilizing aspartate, adding a favorable interaction with the helix dipole,[36] and a phenylalanine was introduced at position 2 to facilitate structural analysis of this region of the helix using NMR spectroscopy.[37] The corresponding reference peptides **nA**, **nG**, and **nP** have an alanine, glycine, or proline at position 3, respectively, and regular oxo-peptide bonds between Phe2 and Ala3 (Table 1).

The CD spectrum of **nΨ** indicates a significant fraction of α-helix (Figure 3A). Comparison with the spectrum of the unsubstituted reference peptide **nA** reveals again a destabilizing effect of the thioxoamide bond. However, the decrease in helical content caused by thioxylation is less pronounced compared to the **cΨ** peptide (cf. Figure 1). As for the central substitutions, the CD spectra of **nΨ** and **nG** peptide are similar in the region

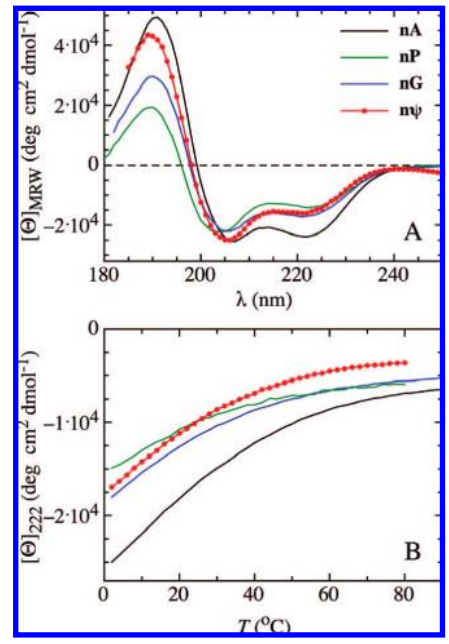


Figure 3. (A) Far-UV CD spectra of the peptides with N-terminal substitutions at 5 °C. (B) Thermal melting of the peptides monitored at 222 nm. Measurements were performed in 10 mM potassium phosphate, pH 7.0.

between 215 and 240 nm, whereas **nP** exhibits significantly less helicity. Also the thermal melting behaviors of **nΨ** and **nG** are similar (Figure 3B) confirming comparable helix contents of these peptides. Based on the ellipticity at 222 nm the helix content of **nΨ** is ~45%, compared to 69% for **nA** (Table 1). Using a w^{thio}-value of 0.044 determined in **cΨ** (Table 1), Lifson−Roig theory predicts 46% helix content for **nΨ**. This agreement indicates that the destabilizing effect of the thioxoamide bond is similar at different positions within the helix. An earlier experimental study on a thioxoamide substituted dimeric GCN4 fragment placed the thioxopeptide group in a widened position in the N-terminal region of a helical coiled-coil.[24] In this case no destabilization was observed, which led us to the conclusion that α-helices can tolerate thioxoamide substitutions without major changes in stability. The discrepancy to our results may arise from the special features of oligomeric coiled-coil structures, which have twisted helices with altered hydrogen-bonding geometry.[25]

NMR Characterization of nΨ. Lifson−Roig theory predicts that the N-terminal three amino acids of **nΨ** have a very low helix propensity (≤16%), whereas the alanine residue following the thioxopeptide linkage (Ala4) populates the helical conformation to ~35% (Figure 2). A plateau of 60% helicity is reached at the residues following the thioxylated peptide bond. The substantial amount of helix formation in the N-terminal part of **nΨ** allows structural investigations around the thioxopeptide bond by recording one- and two-dimensional ^{1}H NMR spectra (Figure 4). The amide protons of **nΨ** give sharp signals in the region from 7.3 to 8.4 ppm, whereas the thioxoamide proton appears as a broadened peak at 9.83 ppm. A downfield shift of this magnitude is generally observed for thioxoamide protons.[6,38] Many of the other resonances overlap, as expected for such a uniform sequence, which prevents a complete assignment based

(35) Doig, A. J.; Chakrabartty, A.; Klingler, T. M.; Baldwin, R. L. *Biochemistry* **1994**, *33*, 3396–3403.
(36) Shoemaker, K. R.; Kim, P. S.; York, E. J.; Stewart, J. M.; Baldwin, R. L. *Nature* **1987**, *326*, 563–567.
(37) Scherer, G.; Kramer, M. L.; Schutkowski, M.; Reimer, U.; Fischer, G. *J. Am. Chem. Soc.* **1998**, *120*, 5568–5574.
(38) Jensen, O. E.; Lawesson, S.-O.; Baadi, R.; Piazzesi, A. N.; Toniolo, C. *Tetrahedron* **1985**, *41*, 5595–5606.

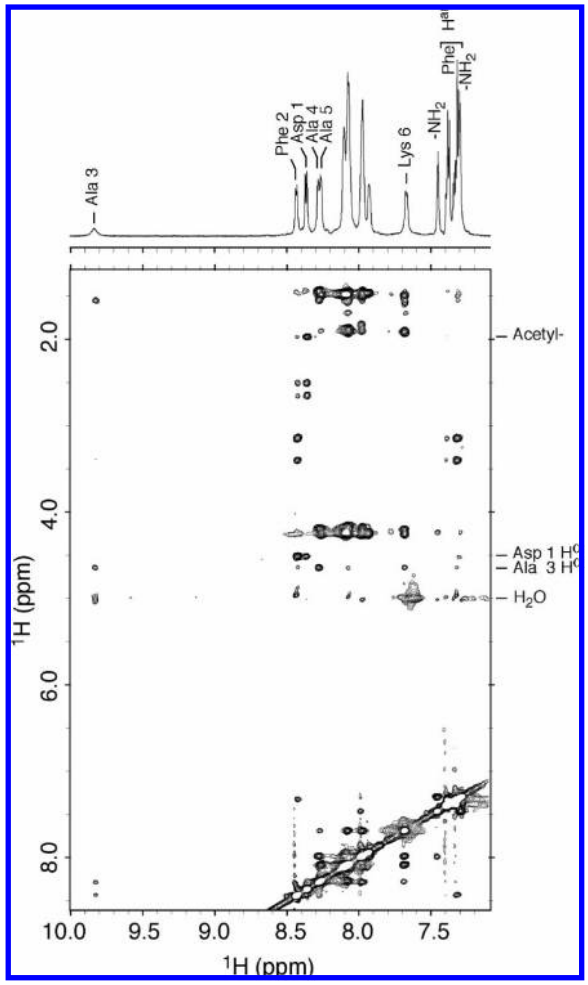


Figure 4. NMR characterization of **nΨ**. The upper panel shows the amide region of the 1D spectrum at pH 7.0 The lower panel displays the corresponding region of the NOESY spectrum recorded with a mixing time of 460 ms at pH 5.0. Some resonances are labelled; the other assignments are listed in Table S1. Measurements were performed in 10 mM potassium phosphate at 2 °C.

on proton spectra, which was achieved for a similar peptide without a destabilizing substitution.[39] However, in the N-terminal region a number of resonances can be assigned using TOCSY and NOESY experiments (Figure 4), since the sequence is less uniform and some resonances are additionally influenced by the sulfur atom and by ring current effects of Phe2 (see Table S2). A key observation in the NMR spectrum is the upfield shift of the Lys6 amide proton ($\delta = 7.68$ ppm). This is due to the influence of the thioamide sulfur atom, since a similar Lys NH upfield shift is absent in the spectra of the all-oxoamide bond containing reference peptides (data not shown). This points to the close vicinity of the sulfur atom and the Lys6 amide proton and makes Lys6 a likely hydrogen bonding partner of the thiocarbonyl group of Phe2. This is supported by the positive temperature coefficient of the Lys6 amide proton resonance ($\Delta\delta/\Delta T = 2.4$ ppb/K) indicating that this proton is involved in a hydrogen bond (Figure S2 and Table S1). In contrast, the amide protons of Asp1, Phe2, Ala3, and Ala4/Ala5 have negative

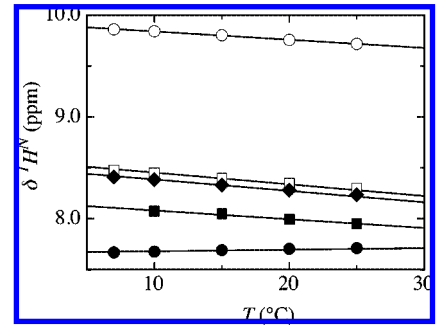


Figure 5. Temperature-dependence of the chemical shifts of the amide and thioxoamide protons in the N-terminal region of **nΨ**. The amide protons of Asp1 (◆), Phe2 (□), Ala4/Ala5 (■), and Lys6 (●) are displayed in addition to the thioxoamide proton of Ala3 (○). Experiments were carried out in 10 mM potassium phosphate, pH 7.0.

temperature coefficients with $\Delta\delta\Delta T \leq -8$ ppb/K (Figure 5 and Table S1), which is typical for non-hydrogen-bonded amide protons.[40,41]

NOE cross-peaks from the thioxoamide proton become detectable upon lowering the pH from 7.0 to 5.0, which slows down thioxoamide proton exchange with water. The chemical shifts of all resonances (Figure 4) as well as the CD spectrum (data not shown) remain unchanged in the range from pH 5 to pH 7. The pattern of NOE cross-peaks in the NOESY spectrum reveals interactions between residues in the region from Phe2 to Lys6 pointing toward helical structure in this region.[42] The well-resolved C^{α}-proton of Ala3 gives particularly useful structural information on the N-terminal region, since it shows cross-peaks to the amide protons of residues Ala3 (*i*), Ala4 (*i*+1), Lys6 (*i*+3), and likely also to the unresolved Ala7 (*i*, *i*+4) (Figure 4). NOEs from Ala3 to Ala5 (*i*+2) are absent, as expected for an α-helical structure, since these residues are on opposite sides of the helix. These observations indicate the formation of an α-helical structure in the region between Phe2 and Lys6. No NOE information can be obtained for the C-terminal region of Lys6 due to spectral overlap of the resonances (Figure 4).

The observed α-helical NOE pattern in the N-terminal region is in agreement with the strong upfield shift of the Lys6 amide proton, caused by the vicinity of the Phe2 thioxoamide group, and with the temperature-dependence of the Lys6 amide proton chemical shift (Figure 5). These findings indicate partial α-helix formation in the N-terminal region of **nΨ** including an *i*, *i*+4 hydrogen bond between the thiocarbonyl group of Phe2 and the amide proton of Lys6.

No evidence for a 3_{10}-helical structure was found for **nΨ**, which was proposed to be generally present at the N-termini of helical peptides[39,43] and was also predicted to be well suited to incorporate the larger sulfur of thioxoamides.[23] Formation of a 3_{10}-helix would result in an *i*, *i*+3 hydrogen bond, i.e., from the thiocarbonyl to the amide proton of Phe2 to Ala5, which can be excluded from the NMR spectra, since the NH proton of Ala5 experiences a similar chemical shift (Figure 4) and a similar temperature-dependence (Figure 5) as the neighboring Ala4.

(39) Millhauser, G. L.; Stenland, C. J.; Hanson, P.; Bolin, K. A.; van de Ven, F. J. *J. Mol. Biol.* **1997**, *267*, 963–974.
(40) Merutka, G.; Dyson, H. J.; Wright, P. E. *J. Biomol. NMR* **1995**, *5*, 14–24.
(41) Cierpicki, T.; Otlewski, J. *J. Biomol. NMR* **2001**, *21*, 249–261.
(42) Wüthrich, K.; Billeter, M.; Braun, W. *J. Mol. Biol.* **1984**, *180*, 715–740.
(43) Bolin, K. A.; Millhauser, G. L. *Acc. Chem. Res.* **1999**, *32*, 1027–1033.

The finding that thioxoamide bonds can be incorporated into α-helical structures confirms the results from measurements on a thioamide substituted S-peptide, which is mainly unstructured in solution but forms an α-helical structure in complex with S-protein (RNase S).[21] A thioxopeptide bond introduced in the N-terminal part of the S-helix had no effect on the catalytic activity of the RNaseS complex. This was interpreted as evidence for helix formation around the thioxopeptide bond.

Conclusions

Introducing thioxopeptide groups at two different positions in alanine-based α-helical peptides reveals a highly helix-destabilizing effect of ~7 kJ/mol, independent of the position within the helix. This can be explained by the longer C═S bond and the larger sulfur atom, which are difficult to integrate into the densely packed α-helix.[23] Additional contributions are likely to arise from the altered hydrogen bonding properties of thioxopeptide groups. The destabilizing effect of a thioxopeptide bond may be different in native protein structures due to tertiary interactions, as observed for alanine-to-glycine substitutions in protein helices compared to peptide models.[44] The NMR data obtained on the peptide with a thioxoamide bond in the N-terminal region of the helix point to the formation of an *i*, *i*+4 hydrogen bond involving the thiocarbonyl acceptor group, whereas no evidence for an *i*, *i*+3 hydrogen bond was observed, which would indicate a 3_{10}-helical structure. This contrasts with the idea that a 3_{10}-helical conformation prevails at the N-terminus of helical peptides,[39,43] which should be even more favored since it is better-suited to accommodate the thioxopeptide bond.[23]

Our results indicate that it is possible to incorporate thioxopeptide groups into α-helical structures despite their strong helix-destabilizing effect. This shows that α-helical regions of proteins can be targets for thioxoamide substitutions in order to test the role of individual backbone hydrogen bonds for protein dynamics and stability. The observed destabilizing effect of an oxo- to thioxoamide bond in the range of 7 kJ/mol is large enough for a reliable determination of protein folding φ-values to test the formation of individual hydrogen bonds in the transition state of a folding reaction or in folding intermediates.[45] In addition, the possibility of fast, light-induced *cis*−*trans* isomerization reactions around thioxopeptide bonds[18–20] should allow the use of thioxoamide groups incorporated into helical structures to trigger fast time-resolved measurement of protein folding reactions.

Materials and Methods

Peptide Synthesis. Peptides were synthesized using standard 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry on an Applied Biosystems 433A synthesizer. Couplings were performed with HBTU/HOBt on Tentagel S RAM resin (Rapp Polymere). Cleavage from the resin and side chain deprotection were achieved with 96/2/2 TFA/TIPS/H_2O (v/v/v). For the synthesis of the thioxoamide substituted peptides a special protocol was used.[46] All peptides were purified to >95% purity by HPLC on an RP C8 column. Purity was checked by analytical HPLC, and the identity was verified by MALDI mass spectrometry.

Concentration Determination. Concentrations of peptides with a thioxoamide bond or a phenylalanine were determined spectroscopically using the extinction coefficients $\varepsilon_{266} = 12\,000\ M^{-1}\ cm^{-1}$ [21] and $\varepsilon_{257} = 197\ M^{-1}\ cm^{-1}$,[47] respectively. A Gly-Tyr sequence was added to the C-terminus of peptides containing neither a phenylalanine nor a thioxo group. The addition of the tyrosine residue facilitates spectrometric concentration determination ($\varepsilon_{275} = 1450\ M^{-1}\ cm^{-1}$) and does not significantly affect the far-UV CD spectrum, when introduced at the C-terminus and separated by a glycine.[30] Concentrations were additionally checked by one-dimensional NMR measurements (see Supporting Information) and differed by less than 5% from the spectroscopically determined concentrations.

CD Measurements. CD measurements were performed on an Aviv DS62 spectropolarimeter. The spectra were independent of the irradiation length, indicating that no significant *trans* to *cis* isomerization of the thioxoamide bond occurred during the measurements. Thermal unfolding transitions were monitored at 222 nm and were fully reversible. Peptide concentrations were between 50 and 100 μM.

NMR Measurements. Proton NMR spectra were recorded on a Bruker DRX 600 spectrometer at 2 °C and referenced to 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) at 0.0 ppm. A detailed description of the one- and two-dimensional experiments is given in the Supporting Information.

Determination of Helix Stability. Lifson and Roig theory[32] was applied as described by Doig et al.[35] to determine the effect of a thioxo substitution on helix stability. The Lifson−Roig parameters for the alanine-based helices were taken from ref 31. The matrix products were calculated without any further approximations using MATLAB 7.2 (MathWorks). To estimate w^{thio}, the *w* of the alanine residue following the thioxoamide bond was adjusted to give the observed helix content.

Acknowledgment. We thank Horst Kessler and Judith Habazettl for discussion and Annett Bachmann for comments on the manuscript. This work was supported by a grant from the Schweizerischen Nationalfonds and by the Munich Center for Integrative Protein Science (CIPSM).

Supporting Information Available: Experimental details of the NMR measurements, CD spectra of the thioxylated peptides in the near-UV CD region, and a table with chemical shift assignments and temperature coefficients. This material is available free of charge via the Internet at http://pubs.acs.org.

JA8015044

(44) Blaber, M.; Zhang, X. J.; Matthews, B. W. *Science* **1993**, *260*, 1637–1640.
(45) Sánchez, I. E.; Kiefhaber, T. *J. Mol. Biol.* **2003**, *334*, 1077–1085.
(46) Wildemann, D.; Drewellow, M.; Fischer, G.; Schutkowski, M. *Chem. Commun.* **1999**, 1809–1810.
(47) Fasman, G. D. *Handbook of Biochemistry and Molecular Biology*, 3rd ed.; CRC Press: 1976; p 186.