Journal of Peptide Science
J. Pept. Sci. 2008; **14**: 1209–1215
Published online 17 July 2008 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.1059

JOURNAL OF PEPTIDE SCIENCE

Short Communication

Switching from the unfolded to the folded state of the helix-loop-helix domain of the Id proteins based on the *O*-acyl isopeptide method

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Received 1 April 2008; Revised 5 June 2008; Accepted 6 June 2008

Abstract: The inhibitors of DNA binding and cell differentiation Id1–4 are helix-loop-helix (HLH) proteins that negatively regulate DNA transcription by forming inactive dimers with ubiquitous and tissue-specific bHLH proteins, including E47 and MyoD, respectively. Their highly conserved HLH domains are essential for heterodimerization, but can also self-associate to highly stable, α-helix-rich structures at low micromolar peptide concentrations. Here, we show that the introduction of an *O*-acyl isodipeptide unit involving the putative *N*-cap serine residue of the *C*-terminal helix completely abrogates the propensity of the Id HLH analogue for any secondary and tertiary structure, resulting in a random coil, as shown by CD measurements in nonbuffered aqueous solutions. However, the HLH fold reappears as soon as an $O \rightarrow N$ intramolecular acyl migration, which occurs spontaneously under physiological conditions, restores the native *N*-cap serine residue. These results show that changes addressing the *N*-terminus of the *C*-terminal helix can dramatically influence the HLH structure, and suggest that local interactions at the junction between the loop and the *C*-terminal helix might be crucial during the HLH folding process. Furthermore, the present study contributes to the evaluation of the *O*-acyl isodipeptide unit as a powerful tool to introduce a conformational switch into peptides.

Keywords: helix-loop-helix motif; Id proteins; *O*-acyl isopeptide; $O \rightarrow N$ acyl migration; conformation switch

INTRODUCTION

Folding and misfolding of peptides and proteins are critical events in the cell, since they determine the normal function or misfunction of these biomolecules in many biologically and pathologically relevant processes. One important class of diseases related to protein misfolding is represented by the amyloidoses that are characterized by the formation of extracellular or intracellular protein deposits rich in β-fibrils [1,2]. The most known amyloidoses are the human neurodegenerative Alzheimer's, Parkinson's and Creutzfeldt–Jakob diseases, which are associated with the misfolding and aggregation of amyloid β peptide (Alzheimer's), tau protein (Alzheimer's and Parkinson's), α-synuclein (Parkinson's) and prion protein (Creutzfeldt–Jakob) [3].

Because of their clinical impact, protein-folding disorders have been attracting great interest that mainly focuses on the understanding of the mechanisms underlying the transformation of soluble proteins with low or no amino acid sequence identity into aggregation-prone, insoluble β-sheet structures. Unfortunately, biophysical investigation of amyloidogenic peptides and proteins is made very challenging by the fact that they strongly aggregate and aggressively precipitate. Hence, peptide and protein chemists have recently put much effort into the development of synthetic strategies aiming to reduce the aggregation propensity of the growing polypeptide chain during solid-phase synthesis, and/or to increase the solubility of the crude product during purification. The most successful approaches rely on the conversion of the peptide bond into a non-*H*-bond donor to weaken interchain contacts, thus reducing peptide aggregation. These include the backbone-amide protection, the pseudoproline and the *O*-acyl isopeptide approaches. The first approach is based on the introduction of amino acid residues bearing a benzyl-type (i.e. 2-hydroxy-4-methoxybenzyl or 2,4-dimethoxybenzyl) protecting group at the α-nitrogen atom that can be removed upon standard acidic cleavage conditions [4]. The pseudoproline strategy consists of the use of serine or threonine residues temporarily masked as pseudoprolines [5,6]. These are then converted into the natural amino acids under standard acidic cleavage conditions. The *O*-acyl isopeptide approach is based on the formation of an ester

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bond during peptide-chain assembly by acylation of the β-hydroxy group of a serine or threonine residue [7–17]. Conversion of the obtained *O*-acyl isopeptide, also referred to as click peptide [12] or switch peptide [8], to the native peptide by $O \rightarrow N$ intramolecular acyl migration occurs spontaneously at neutral or slightly basic pH values. However, as the isopeptide structure is retained under standard acidic cleavage conditions, the crude product can be purified in its more soluble and less aggregation-prone form, which makes the purification step more successful.

Besides its wide application in the synthesis and purification of difficult peptide sequences (e.g. amyloid β peptide 1–42 and its analogues [10,12–14,17,18], or the Asn-15 analogue of the WW domain FBP28 [19]), another elegant application of the *O*-acyl isopeptide method has been made in the control of peptide conformation and aggregation. For example, Mutter and coworkers developed *O*-acyl isopeptides based on amyloid β peptide sequences to disrupt and induce secondary structure and self-assembly [8,20,21]. Switch peptides have been successfully used by Börner and coworkers also for peptide-directed polymer microstructure formation [22–26]. As the *O*-acyl isopeptide approach allows not only to modify the peptide backbone, but also to make such modification restorable to the native peptide bond, it can be used to examine conformational transformations of peptides from a nonnatural to a natural state. Herein we present the application of this approach in the study of the helix–loop–helix (HLH) structural motif of Id1 [27–29] and Id2 [30–33], two inhibitors of DNA binding and cell differentiation that negatively regulate basic-HLH (bHLH) transcription factors. The biological function of Id1, Id2, and of the other mammalian members of the Id protein family (Id3 and Id4) is mostly dictated by their highly conserved, 41-residue long HLH domain that is essential for Id protein dimerization with ubiquitous (e.g. E12 and E47) and tissue-specific (e.g. MyoD) bHLH transcription factors [34–36] (Figure 1). These Id-bHLH dimers are not able to bind the DNA because the Id subunit, contrarily to the bHLH one [37], lacks the basic region required for DNA interaction. As a result, the Id proteins negatively regulate bHLH-mediated transcriptional activation. Id protein activity usually correlates with cell hyperproliferation and dedifferentiation, and plays a key role during development and tumorigenesis [38–40]. Thus far, no crystal or solution structure of the Id proteins has been reported in the literature; however, insights in the conformation of the Id HLH domains have been provided by homology models based on known structures of related bHLH proteins [41,42]. Moreover, CD studies of synthetic peptides reproducing the native Id HLH sequences or amino-acid mutated/deleted analogues have been carried out [43–45]. These studies have shown that the Id HLH domain is highly helical and self-associates at low micromolar peptide concentrations: indeed, the Id1 HLH domain was found to be predominantly in the disordered, monomeric form only at submicromolar concentrations and a temperature of at least 60 °C, as reported by Fairman *et al.* [43]. In the present study, we show that, by converting the Id HLH domain into an *O*-acyl isopeptide analogue based on a conserved serine residue at the loop–helix-2 junction, the otherwise highly stable helical fold is completely lost, but it can be fully restored by inducing an $O \rightarrow N$ intramolecular acyl migration.

MATERIALS AND METHODS

Synthesis of Boc-Ser(Fmoc-Val)-OH

Boc-Ser(Fmoc-Val)-OH was synthesized by following the procedure previously reported by Kiso and coworkers [46,47]. Briefly, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (104 mg, 0.54 mmol) was added to a solution of Boc-Ser-OBn (133 mg, 0.45 mmol), Fmoc-Val-OH (183 mg, 0.54 mmol) and 4-(dimethylamino)-pyridine (5.5 mg, 0.045 mmol) in dry chloroform under argon atmosphere and at 0 °C. The mixture was left to warm up to room temperature and stirred overnight. After evaporation of the solvent, ethyl acetate was added and the solution was washed successively with water, 1 M HCl, water, saturated $NaHCO_3$ and brine. The organic layer was dried over Na_2SO_4 and the solvent was removed *in vacuo*. The crude compound was purified

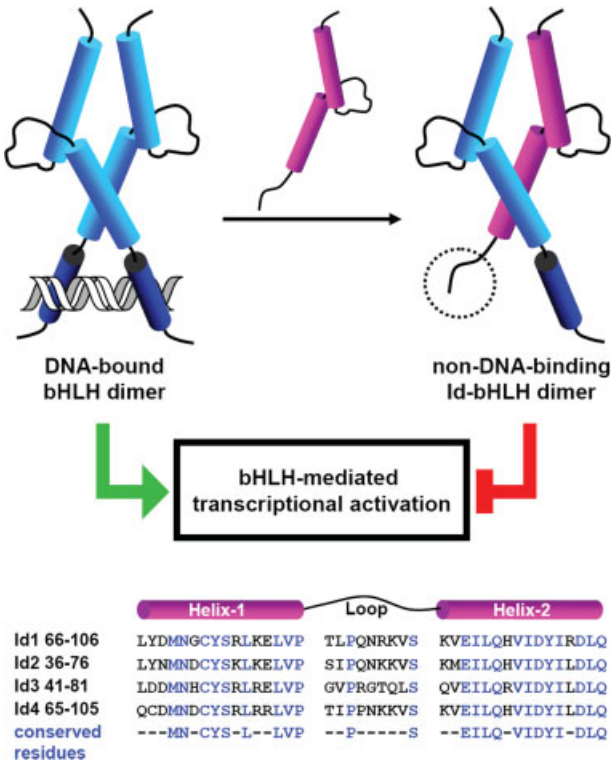


Figure 1 Negative regulation of bHLH transcription factors by the Id proteins (top). Amino acid sequences of the human Id HLH domains (bottom).

DOI: 10.1002/psc

by column chromatography (ethyl acetate/hexane 1 : 4 v/v). Yield: 69%; mass calcd./found (Da): 616.28/639.10 ($M + Na^+$) [47]. Successively, the benzyl group was cleaved overnight by catalytic hydrogenation in ethanol using Pd/C (20 mg) under hydrogen atmosphere. After removal of the catalyst by filtration over celite, the crude compound was purified by column chromatography (at first with ethyl acetate, and finally with methanol). Yield: 83%; mass calcd./found (Da): 526.23/549.00 ($M + Na^+$) [47].

Solid-phase Peptide Synthesis

The synthesis of the native HLH peptides Id1 66–106 and Id2 36–76 (*N*-terminally acetylated and *C*-terminally amidated) was described previously [44]. The corresponding *O*-acyl isopeptide analogues were obtained by manual elongation of the fragments Id1 91–106 and Id2 61–76 that were automatically assembled by following a synthetic protocol reported previously [44]. Briefly, Id1 91–106 and Id2 61–76 were prepared on a 0.018 mmol scale on an automated multiple peptide synthesizer (Syro I, MultiSynTech, Witten, Germany). Side-chain protecting groups of the Fmoc-amino acids were as follows: *tert*-Bu for Asp, Glu, Ser, Thr, and Tyr, Boc for Lys, trityl for Cys, His, Asn, and Gln, 2,2,4,6,7-pentamethyldihydro-benzofurane-5-sulfonyl or 2,2,5,7,8-pentamethylchromane-6-sulfonyl for Arg. Chain assembly was accomplished on Rink-amide-MBHA (4-methyl-benzhydryl-amine) resin (loading: 0.64 mmol/g, purchased from Novabiochem, Merck Biosciences GmbH, Schwalbach/Ts., Germany) by a double coupling procedure (2×40 min) with the mixture Fmoc-amino acid/1-[bis(dimethylamino)methylene]-1*H*-benzotriazolium hexafluorophosphate 3-oxide (HBTU)/HOBt/DIPEA (5 : 5 : 5 : 10 equiv.) in DMF/NMP (70 : 30, v/v), followed by Fmoc removal with 40 and 20% piperidine in DMF/NMP (80 : 20, v/v) for 3 and 10 min, respectively. The *O*-acyl isodipeptide Boc-Ser(Fmoc-Val)-OH was attached manually by single coupling (2 h) with the mixture Fmoc-isodipeptide/*N*,*N*′-diisopropylcarbodiimide/HOBt (5 : 5 : 5 equiv.) in dry dichloromethane, followed by Fmoc cleavage with 20% piperidine in DMF for 8 min. The coupling/deprotection protocols used for the isodipeptide were also applied for peptide-chain elongation with the remaining amino acids, with the exception that DMF was used as solvent for the coupling step. Isopeptide cleavage from the resin with simultaneous side-chain deprotection was obtained by treatment of the isopeptidyl resin with TFA/water/triisopropylsilane/1,2-ethanedithiol (90 : 5 : 2.5 : 2.5, v/v) for 3 h. The crude isopeptides were then precipitated from ice-cold diethylether, recovered by centrifugation at 3 °C, and finally purified by preparative RP-HPLC (Agilent equipment, Böblingen, Germany) using the Phenomenex Luna C18(2) column, 10 μm, 100 Å, 21.2×250 mm (Aschaffenburg, Germany). The purified products were characterized by analytical RP-HPLC (L-6200A Intelligent Pump from Merck, HP detector Series 1050 from Agilent) using the Phenomenex Luna C18(2) column, 3 μm, 100 Å, 4.60×150 mm, and by MALDI-TOF mass spectrometry (Future GSG spectrometer, Bruchsal, Germany). Isopeptide purity (based on analytical HPLC) >90%. $MW_{calcd.}/MW_{found}$(Da) : *iso*-Id1 HLH, 4931/4932 ($M + H^+$); *iso*-Id2 HLH 4907/4908 ($M + H^+$).

CD Spectroscopy

Each peptide was dissolved in water as well as in phosphate buffer (0.1 M, pH 7.3). Peptide concentration was determined spectrophotometrically by measuring the tyrosine absorbance at 280 nm ($\varepsilon = 1480\ M^{-1} \cdot cm^{-1}$ for each tyrosine residue [48]). The CD measurements were recorded at room temperature on a Jasco J-710 spectropolarimeter using a quartz cell with a path length of 0.02 cm. For each CD spectrum, five scans were accumulated using a step resolution of 0.2 nm, a bandwidth of 2 nm, a response time of 2 s, and a scan speed of 20 nm/min. The CD spectrum of the solvent was subtracted from that of the sample to eliminate interferences from cell, solvent, and optical equipment. Noise reduction was performed with a Fourier transform filter from the Origin program (OriginLab Corporation, Northampton, MA, USA). The ellipticity is expressed as mean residue ellipticity $[\Theta]_R$ in $deg \cdot cm^2 \cdot dmol^{-1}$.

RESULTS AND DISCUSSION

The amino acid sequences of the four Id HLH domains are highly conserved, especially within the *N*-terminal and *C*-terminal helix segments (Figure 1). The *N*-terminal helix-1 is *C*-terminally broken by the presence of a conserved proline residue that represents the junction between helix-1 and the loop. The latter spans a nine-residue sequence with a conserved proline residue at third position and a serine residue at the junction with helix-2. Interestingly, this serine residue may be seen as the *N*-cap position of the canonical helix-capping box consisting of the motif S/T-X-X-E at the *N*-terminus of an α-helix, in which the α-amide proton and β-hydroxy group of serine or threonine are involved in *H*-bonds with the γ-carbonyl oxygen and α-amide proton of glutamic acid, respectively (Figure 2) [49]. As such, the serine residue might be an important stabilizing element for Id helix-2, and, consequently, for the whole Id HLH fold.

The *O*-acyl isopeptide approach provides a valuable tool to investigate the role of serine and threonine in peptide folding, as it introduces a double modification consisting of the conversion of the α-amide into a free amine and of the β-hydroxy group into an ester group. Therefore, we chose this method to examine the impact of these changes on the putative *N*-cap serine of the Id HLH domain. Because of the presence of the S-X-X-E sequence motif in all four members of the Id family (Figure 1), we selected just two of them, Id1 and Id2, to carry out the study. The native Id1 and Id2 HLH peptides and the corresponding *O*-acyl isopeptide analogues were synthesized by solid-phase methodology using Fmoc-chemistry (Scheme 1). The *O*-acyl isodipeptide Boc-Ser(Fmoc-Val)-OH was used as a building block to give the corresponding *O*-acyl isopeptide structure. After preparative HPLC purification, the final peptides were characterized by mass spectrometry and analytical HPLC (Figure 3).

DOI: 10.1002/psc

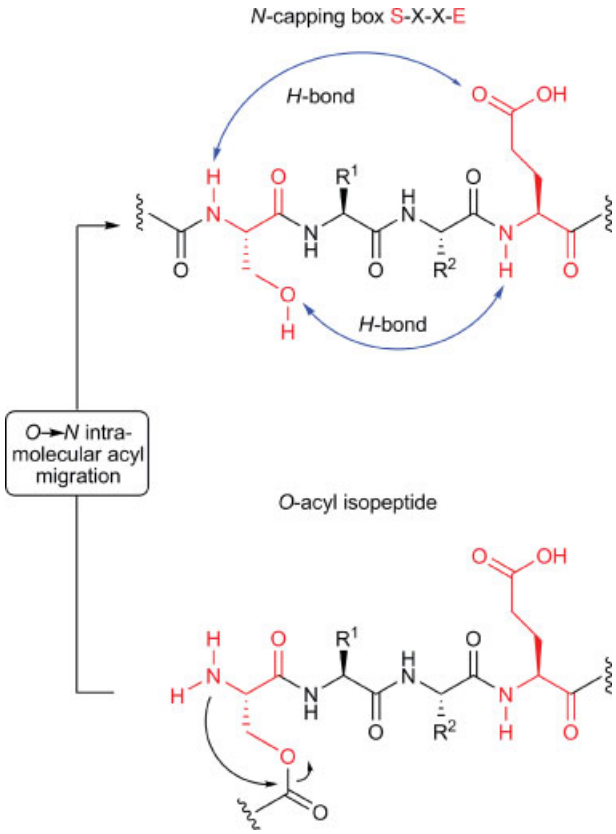


Figure 2 Side-chain-to-backbone *H*-bonding pattern in the canonical helix *N*-capping box S-X-X-E (top). An *O*-acyl isopeptide analogue (bottom) is converted back to the native form by spontaneous $O \rightarrow N$ intramolecular acyl migration at neutral or slightly basic pH values.

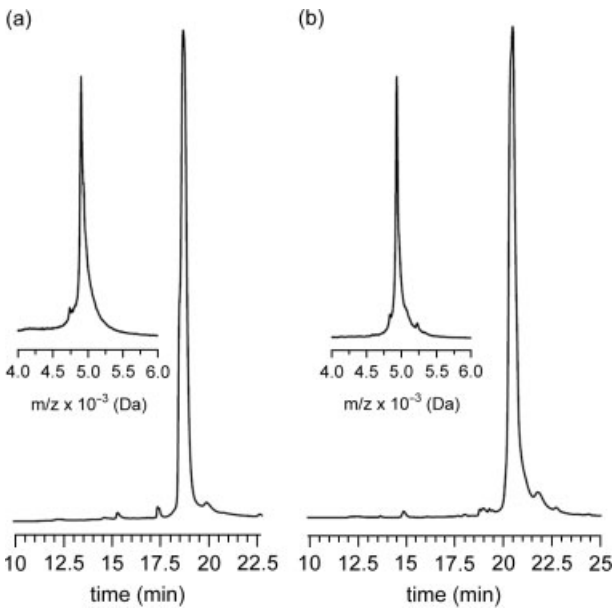
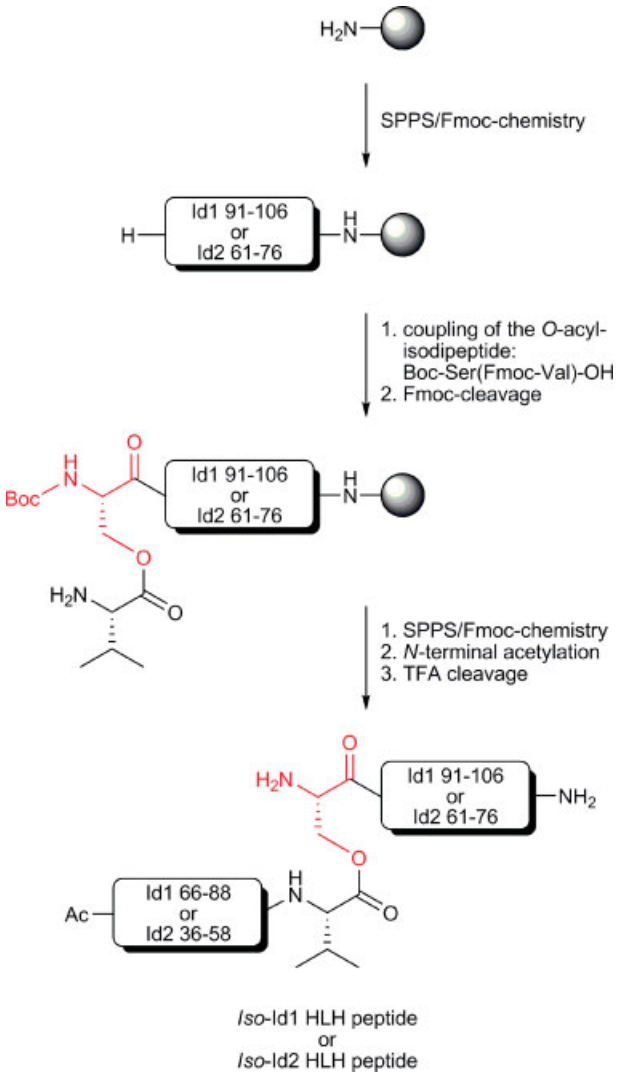


Figure 3 RP-HPLC profiles and MALDI-TOF-MS spectra of the purified peptides *iso*-Id1 HLH (a) and *iso*-Id2 HLH (b).



Scheme 1 Synthesis of the *O*-acyl isopeptide analogues of the HLH domains of Id1 and Id2.

The conformation of the *O*-acyl isopeptides, *iso*-Id1 HLH and *iso*-Id2 HLH, was investigated in nonbuffered aqueous solutions (pH 4.5) to prevent the $O \rightarrow N$ intramolecular acyl shift. For comparison, the corresponding native Id1 and Id2 HLH motifs were also measured in nonbuffered aqueous solutions (pH 4.5). Although more stable at neutral pH values, the conformation of the native Id HLH peptides is α-helical at acidic pH values too, as shown in Figure 4. In contrast, the corresponding *O*-acyl isopeptides do not present any secondary structure preference at pH 4.5, as deduced by the shape of their CD spectra that is characteristic of disordered peptides (red-colored curves in Figure 5). No time-dependent conformational changes were observed for the samples that gave overlapping CD spectra 5 min up to 3 days after sample preparation (data not shown). These results indicate that the backbone and side-chain modifications introduced at the putative *N*-cap serine residue cause a complete loss of the native structure,

DOI: 10.1002/psc

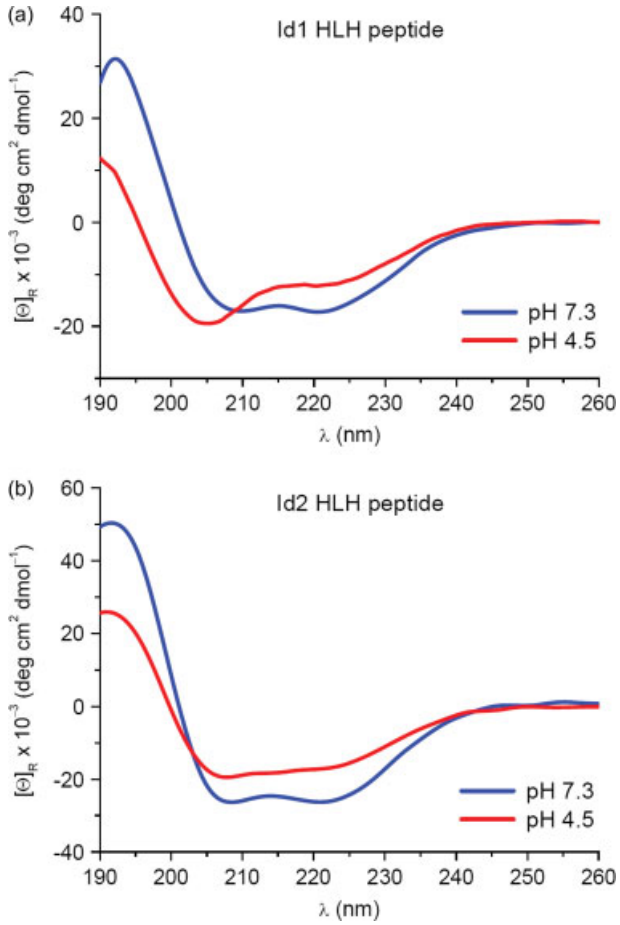


Figure 4 CD spectra of the native Id1 (a) and Id2 (b) HLH domains (30 μM) in nonbuffered (pH 4.5) and buffered (0.1 M phosphate buffer, pH 7.3) aqueous solutions at room temperature.

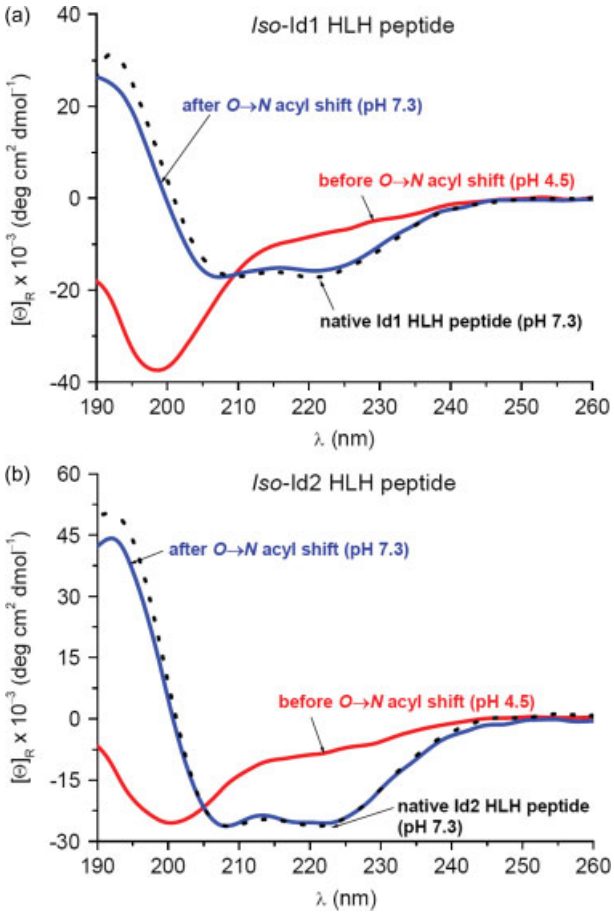


Figure 5 CD spectra of *iso*-Id1 (a) and *iso*-Id2 (b) HLH peptides before and after $O \rightarrow N$ acyl shift (read text for details).

which is accompanied by a conformational change from a highly ordered α-helical fold to a random coil. The dramatic effect of such chemical changes on the structural preferences of the Id HLH sequences is likely to arise from the fact that favorable *H*-bonds involving the α-amide proton as well as the β-hydroxy group of serine are suppressed in the *O*-acyl isopeptide analogues. In addition, the protonated α-amino group of serine in proximity of the *N*-end of helix-2 might contribute to the helix destabilization by repulsive interaction with the helix-2 macrodipole. However, converting the incorporated *O*-acyl isodipeptide unit into the corresponding dipeptide one is expected to restore the native helix fold. Thus, the two *O*-acyl isopeptides were dissolved in phosphate buffer at pH 7.3 to induce the $O \rightarrow N$ acyl shift generating the native peptide bond. As expected, the two buffered peptide solutions show CD spectra that are not only characteristic of helical structures (blue-colored curves in Figure 5), but also super-imposable to those of the native Id HLH peptides (black-colored dotted curves in Figure 5). This indicates that restoring both the α-amide and β-hydroxy groups of serine leads to a complete recovery of the Id HLH fold.

Previous studies have suggested that the *C*-terminal helix-2 of the Id HLH domain possesses more pronounced helical character than the *N*-terminal helix-1 [44,45]. On the basis of the presented results, the fact that altering the *N*-terminal capping box of helix-2 leads to loss of secondary and tertiary structure suggests that formation of a stable helix-2 triggers the folding of the whole Id HLH domain. However, as the *O*-acyl isodipeptide unit is located at the junction between the loop and helix-2, its negative effect on the Id HLH structural motif might reflect not only a destabilization of helix-2, but also an alteration of local interactions between the *C*-terminal loop residues and the *N*-terminal helix-2 ones, which could be crucial for keeping helix-2 correctly oriented in the fold. Indeed, it has been reported that hydrophobic interactions between the residue *i* located before the *N*-cap and the one at position $i+5$ of a helix in peptide models and proteins are important to better define not only the beginning of the helical segment, but also its orientation with respect to the preceding segment [50]. Therefore, in the case of the

DOI: 10.1002/psc

Id HLH domains, besides the helix-capping box S-X-X-E, the residue located before serine (valine or leucine) and the one located after the glutamic acid (isoleucine) might interact with each other by hydrophobic contacts, thus contributing to the HLH folding either by further stabilizing the *N*-end of helix-2 and by inducing a peptide-chain reversal that would allow for the interaction of helix-2 with other loop and, more importantly, helix-1 residues.

CONCLUSIONS

The Id HLH peptides fold into highly stable, self-associating, α-helix-rich structures [43,44]. Besides the highly conserved positions within the *N*-terminal and *C*-terminal helix segments, a conserved serine residue is present at the *N*-cap position of the *C*-terminal helix-2. Modification of this serine by incorporation of an *O*-acyl isodipeptide building block resulted in unstructured Id HLH analogues. However, these were converted back to their native, folded counterparts by allowing an $O \rightarrow N$ intramolecular acyl migration under physiological conditions. These results suggest that local interactions occurring at the junction between the loop and helix-2 may be crucial for the formation of secondary and tertiary structure within the HLH regions of the Id proteins. Further efforts will be made in the near future to better define the role of the residue side chains as well as backbone amides connecting the loop with helix-2. Moreover, it will be important to investigate the conformational properties of a number of Id HLH analogues containing the *O*-acyl isodipeptide unit at different positions (i.e. at the junction between helix-1 and the loop), in order to identify other key structural motifs and elucidate their participation to the folding and self-association processes of the Id HLH domains.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft (Emmy-Noether grant CA296 to C.C.). S.D.K. is grateful to the Japanese Society for the Promotion of Science for a fellowship. The referees of the manuscript are gratefully acknowledged for their careful reading and helpful comments.

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DOI: 10.1002/psc