Asn^{78} and His^{81} form a destabilizing locus within the Max HLH-LZ homodimer

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Abstract The human Max protein lies at the center of the Myc/Max/Mad family of transcription factors. Its role at the center of this regulatory network is dependent on the helix-loop-helix leucine zipper (HLH-LZ) dimerization domain. The Max LZ contains three residues that deviate from the pattern of hydrophobic amino acids normally present at the interface of LZ dimers: Asn^{78}, His^{81} and Asn^{92}. In contrast to interfacial Asn residues in other LZ proteins, we have shown that Asn^{92} does not act to destabilize the homodimer. Here we describe thermal denaturation experiments performed on Asn^{78} and His^{81} mutants demonstrating that these residues are involved in actively destabilizing the Max homodimer. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Coiled-coil; Helix-loop-helix; Leucine zipper; Max; c-Myc; Protein–protein interaction

1. Introduction

The human Max protein is the central figure in a network of transcription factors that play a vital role in the control of cellular growth, proliferation, differentiation and death (for reviews see [1–5]). Max was identified by Blackwood and Eisenman [6] as the obligate dimerization partner of the Myc oncoprotein, and has been shown to be essential for the DNA binding, transcription activating, transforming and apoptotic properties of Myc [7]. In addition to heterodimerizing with Myc, Max forms homodimers that are transcriptionally inert [8,9]. The Max associated network of transcription factors has since enlarged. New proteins have emerged that interact with Max: the Mad family of proteins, Mnt, Mlx, TEF-1 and Mga [10–18]. Max forms homo- and heterodimers through the helix-loop-helix leucine zipper (HLH-LZ) motif common to each member of this family of transcription factors.

The LZ is a dimerization motif characterized by a heptad repeat of leucine residues [19,20]. Folded dimers form a parallel, coiled-coil of α-helices in which, by conventional nomenclature, $(abcdefg)_n$, the heptad repeat Leu residues occupy position *d* at the dimer interface. Leu is highly conserved at position *d* within LZ proteins, whereas β-branched hydrophobic amino acids, such as Ile and Val, are conserved at position *a*. Hydrophobic interactions at *a* and *d* position residues form the interface of the amphipathic coiled-coil, whilst charged residues occupy positions *e* and *g* on the external surface of the dimer.

The crystal structure of bHLH-LZ domains of Max showed them interacting as a parallel, left-handed four-helix bundle [21]. Each monomer consists of two right-handed α-helices joined by the loop region of the HLH. The first α-helix consists of the basic DNA binding region contiguous with helix 1 of the HLH domain, and the second α-helix consists of helix 2 of the HLH domain continuous with the LZ. The LZ extends as a coiled-coil out of the globular HLH domain. Horiuchi et al. [22] have shown that the Max bHLH-Z domain homodimerizes cooperatively in a manner consistent with a two-state, monomer to dimer association. There is also evidence that the Max HLH-LZ is able to interact as a tetramer under certain conditions [23,24].

The Max LZ exhibits a number of non-classical residues at the dimer interface: an *a* position Asn at residue 78, a *d* position His at residue 81, and an unusual Gln/Asn tetrad arrangement at residues 91 and 92. The ^{1}H-NMR solution structure of the Myc/Max LZ heterodimer shows that these three regions are favorably buried at the dimer interface. These residues play important roles in the specificity of interaction between Myc and Max through salt bridges and H-bonds across the LZ interface [25].

The majority of LZ proteins contain a single *a* position Asn residue that confers dimer specificity at the expense of stability and higher order oligomer formation. Mutation of this interfacial polar residue to a Val residue in the GCN4 LZ dramatically stabilizes the coiled-coil and promotes the formation of higher order oligomers [26]. NMR studies of a recombinant Jun LZ peptide showed that its interfacial Asn residue is hydrogen bonded (H-bonded) and adopts two distinct, rapidly exchanging conformations in solution [27]. Mutation of this Asn to a Leu residue caused changes in dimer stability and oligomerization status comparable to the behavior of the Asn to Val mutant of GCN4. Presumably, these destabilizing polar residues are conserved across the LZ family because they play an important role in facilitating dimer exchange between the various LZ members of each transcription factor network.

In contrast to the results described for GCN4 and Jun, we have recently shown that Asn^{92} within the Max LZ does not destabilize the homodimer [28]. The native homodimer displays comparable stability to that shown by an Asn to Val mutant. Considering the central role of Max within its network of bHLH-LZ transcription factors, destabilization of the homodimer is likely to be important in rapidly facilitating

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Abbreviations: HLH, helix-loop-helix; LZ, leucine zipper; CD, circular dichroism

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Max exchange from the homodimer to heterodimer forms, in response to the presence of its various dimerization partners. Having discounted Asn[92] as being responsible for this destabilizing influence, we have examined His[81] and Asn[78] as potential regions of the Max LZ that could fulfill this role. Both of these residues occupy positions at the interface of the LZ and are, therefore, suitably positioned to disrupt hydrophobic interactions within the homodimer. His[81] occupies a *d* position within the LZ and Asn[78] is situated N-terminal to His[81] in an *a* position.

2. Materials and methods

2.1. Cloning and mutagenesis

A synthetic gene was designed which encoded the HLH and LZ domains of the human Max protein (amino acids 37–105[i]) with an additional Gly-Gly-Cys at the C-terminus. *Bam*HI sites were incorporated onto the 5′ and 3′ ends of the coding sequence to permit insertion into the pGEX-2T expression vector [29]. The final DNA sequence was optimized for bacterial expression by replacing rare codons with those frequently used in *Escherichia coli* [30]. pGEX-MaxN (for native Max) was isolated by expression screening, and its identity confirmed by automated DNA sequencing in both strands.

Mutagenic PCR [31] was used to introduce changes into pGEX-MaxN, yielding pGEXMax vectors with mutations at His[81] and Asn[78] (pGEXMaxH81Y, pGEXMaxH81A, pGEXMaxH81L, and pGEX-MaxN78V). Mutagenic primers used to introduce the indicated alterations were: H81Y (5′-AACCACACC**TAC**CAGCAGGAC-3′ and 5′-GTCCTGCTG**GTA**GGTGTGGTT-3′), H81A (5′-AACCACACC-**GCT**CAGCAGGAC-3′ and 5′-GTCCTGCTG**AGC**GGTGTGGTT-3′), H81L (5′-AACCACACC**CTG**CAGCAGGAC-3′ and 5′-GTCC-TGCTG**CAG**GGTGTGGTT-3′), N78V (5′-CGTCGTAAC**GTT**CA-CACCCAC-3′ and 5′-GTGGGTGTG**GTT**CTTACGACG-3′).

Non-complementary bases that introduced mutations are indicated in bold. Each round of mutagenic PCR also used the following common primers at the 5′ and 3′ ends of the Max gene: 5′-GGATCC-GACCACATCAAAGACTCCTTC-3′ and 5′-GGATTCTAATAGT-GATCACTATTAGCAAC-3′.

2.2. Protein production

Each GSTMax fusion protein was overexpressed in *E. coli* DH5α. Cells were induced with 0.5 mM IPTG and grown at 37°C for 3 h prior to harvesting. GSTMax fusion proteins were expressed into both the soluble and insoluble phases of the cellular lysate under these conditions. Soluble GSTMax fusion proteins were separated from cellular proteins by glutathione agarose affinity chromatography and Max proteins were released from the fusion protein at the engineered thrombin cleavage site with bovine thrombin. Max proteins were purified by HPLC on a semi-preparative Vydac C-18 column with a linear gradient from 25 to 45% acetonitrile 1% (v/v) TFA, over 30 min. Following HPLC, the protein preparations used in this study were confirmed to be pure by tricine gel electrophoresis and of the correct molecular weight by ESMS.

2.3. Circular dichroism (CD) measurements

Each homodimer was diluted from stock solutions into physiological ionic salt (PIS) buffer (50 mM Tris–HCl, 125 mM NaCl, 1 mM EDTA), and sample pH adjusted appropriately with concentrated HCl or 10 M NaOH. Sample pH was confirmed on a Cyberscan500 pH meter (Selby Biolabs, Clayton, Vic., Australia 3168) and the volume corrected such that the final peptide concentration was 0.1 mg/ml. Stock peptide concentrations were determined by UV absorbance at 280 nm (A_{280}) using the extinction coefficient value of 2620 cm^{-1} M^{-1} for each Max peptide, except MaxH81Y which has an extinction coefficient of 3900 cm^{-1} M^{-1}. Far ultraviolet CD spectra were collected on a Jasco J-720 spectropolarimeter flushed continuously with N_2 and routinely calibrated with D-(+)-camphor-10-sulfonic acid. For thermal denaturation profiles, ellipticity at 222 nm for each of the disulfide bridged homodimers was measured over a linear temperature gradient from 10 to 95°C at 1°C/min. Each thermal denaturation experiment was performed three times and the mean calculated to give the final profile. Thermal denaturation profiles of MaxH81A and MaxH81Y were performed at pH 5.1, 7.4 and 9.8. The thermal denaturation profile of MaxN was obtained at pH 5.1, 5.6, 6, 6.8, 7.4, 8.8 and 9.8. MaxH81L and MaxN78V were studied at pH 7.4 only. Baseline spectra of PIS buffer were collected and subtracted from all data prior to conversion into mean residue weight ellipticity values using the formula:

$$[\theta] = \frac{\theta \cdot 100 \cdot \mathrm{MRW}}{c \cdot d} \quad (1)$$

where mean residue weight ellipticity ($[\theta]$) is expressed in deg cm^{-2} $dmol^{-1}$, θ is raw ellipticity value, MRW is the molecular weight divided by the number of residues in the peptide, c is the peptide concentration in mg/ml, and d is the pathlength in cm through the optical cell [32]. Each thermal denaturation profile was fitted to the following sigmoidal function:

$$[\theta]_{222} = a + bT - \{(c + dT) - (a + bT)\}\left\{\frac{\exp(m(T - T_m))}{1 + \exp(m(T - T_m))}\right\} \quad (2)$$

3. Results

Thermal denaturation of disulfide bridged homodimers was followed by measurements of $[\theta]_{222}$ from 10 to 95°C. Decreases in absolute $[\theta]_{222}$ correlate with decreasing α-helicity and the formation of monomeric peptides from the initial homodimers [28]. Each protein underwent a decrease in helicity and a shift to an increasingly random structure as it was heated. Max proteins displayed a cooperative mechanism of unfolding as evidenced by the sigmoidal shape of each thermal denaturation curve.

Initial $[\theta]_{222}$ measurements indicated that the basal helicity of the His[81] mutant peptides was less than that of MaxN (Fig. 1). Minor differences in $[\theta]_{222}$ values were likely to be due to small errors in calculating peptide concentration, but this did not affect the overall shape of the curve. The resulting T_m values are more accurate measurements of dimer stability than are absolute $[\theta]_{222}$ values [28].

At pH 7.4, MaxN showed a cooperative unfolding event with a melting temperature (T_m) of 56°C, where T_m is defined as the temperature at which half the peptide population is folded. The thermal denaturation profile exhibited good correlation to the model curve (Eq. 2). MaxH81A and MaxH81Y also unfolded cooperatively, with melting temperatures of 61

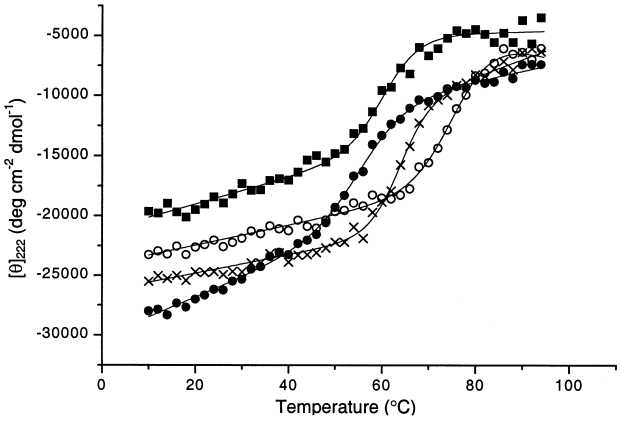

Fig. 1. Thermal denaturation curves of MaxN (●), MaxH81A (■), MaxH81Y (×) and MaxH81L (○) were followed by CD spectroscopy at 222 nm ($[\theta]_{222}$). The midpoint of each fitted curve (T_m) correlates to the thermal stability of the disulfide bridged homodimers. The T_m values of MaxN, MaxH81A, MaxH81Y and MaxH81L were 56, 61, 64 and 76°C, respectively.

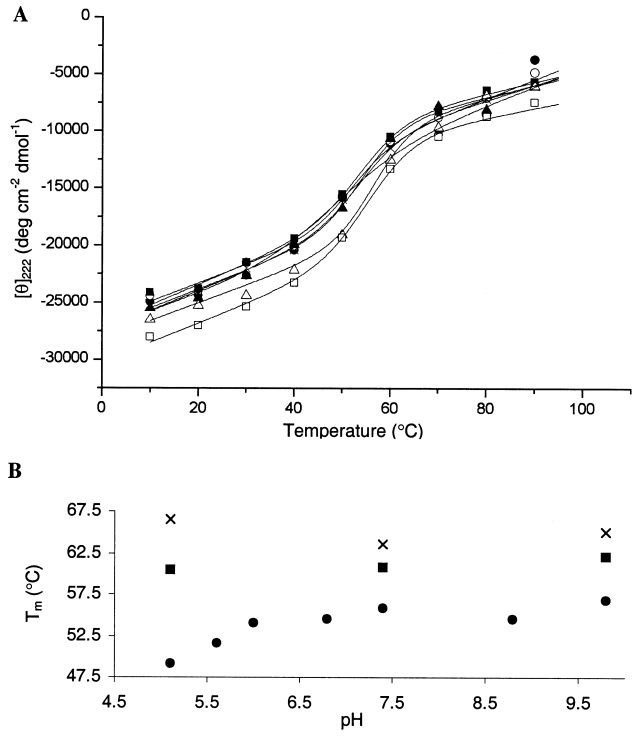

Fig. 2. A: Thermal denaturation curves of MaxN at pH 5.1 (●), 5.6 (○), 6.0 (×), 6.8 (■), 7.4 (□), 8.8 (▲), and 9.8 (△). B: Plot of melting temperature (T_m) versus pH for MaxN (●), MaxH81A (■) and MaxH81Y (×). Extremes of pH did not produce any significant changes in T_m to MaxH81A or MaxH81Y. In contrast, MaxN was destabilized at pH values below 6.8.

and 64°C, respectively. Replacement of His^{81} by a Leu residue in MaxH81L resulted in a larger increase in T_m to 76°C. The increases in thermal stability associated with replacement of His^{81} showed that this residue destabilizes the Max homodimer (Fig. 1).

Analysis of the T_m of MaxN over a range of pH values showed that the native peptide is destabilized at low pH. Below pH 6.8, the T_m of MaxN steadily decreased to a minimum value of 49°C at pH 5.1. In contrast, the melting temperatures of MaxH81A and MaxH81Y were unaffected by changes in pH (Fig. 2). Thus, the decreased thermal stability of MaxN at low pH is unlikely to be due to the dominant influence of other residues, but is specifically due to protonation of His^{81}. Furthermore, the destabilization of MaxN at low pH values indicated that His^{81}, although it is positioned at the dimer interface, remains accessible to solvent.

MaxN78V also showed a cooperative mechanism of unfolding that fitted well to the theoretical curve. MaxN78V unfolded at a T_m of 64°C, significantly higher than native MaxN (Fig. 3). Hence, replacement of the native Asn^{78} with a branched hydrophobic residue at this position in the LZ stabilized the homodimer.

4. Discussion

Stabilization of the native Max homodimer was achieved by replacement of His^{81} with Ala, Tyr and Leu residues. The 20°C increase in thermal stability achieved by replacement of His^{81} with a Leu residue was expected. Leu residues are highly conserved at the *d* position within LZ proteins [33] and are probably needed to maximize stabilizing hydrophobic contacts at the dimer interface.

Replacement of His^{81} with an Ala residue increased the stability of the homodimer by 5°C. The Ala replacement represents a 'neutral' or 'negative' mutation, that is, its sidechain has neither bulk nor charge and hence imparts little positive effect. Thus the modest improvement in thermal stability of MaxH81A over MaxN indicated that either the size or polar nature of His^{81} was involved in actively destabilizing the homodimer. Additionally, Ala residues are known to have a greater helical propensity than His sidechains in coiled-coil systems [34], hence the modest increase in thermal stability of the maxH81A mutant was not unexpected.

Tyr is found as a *d* position residue within the LZ of Mlx [15,17]. Our choice of Tyr as a substitute residue for His^{81} was influenced by this naturally occurring precedent. The sidechain of Tyr is approximately the same size as His, and although Tyr does contain an -OH group at its extremity, it is more hydrophobic than the imidazole ring of His and so might be expected to fit more comfortably at the hydrophobic interface. The resulting 8°C increase in T_m supported this hypothesis, indicating that it is not only the bulky cyclical nature of the imidazole ring that destabilizes the homodimer at this location; the energetically unfavorable solvation of the polar or charged imidazole group also acts in a destabilizing manner.

It is interesting to note that the initial slope of the MaxN thermal denaturation profile indicated that it undergoes a gradual partial unfolding process prior to the cooperative event at 56°C. In contrast, the His^{81} mutant forms exhibited flatter baselines, indicating that in addition to having greater thermal stability, the mutant forms were better able to maintain helicity as the proteins were heated. This supports the suggestion that His^{81} promotes unfolding of the HLH-LZ, whereas the three His^{81} mutants act to stabilize the homodimer.

Thermal denaturation studies at a number of pH values showed that His^{81} is accessible to solvent and may be protonated under acidic conditions. The T_m of both MaxH81A and MaxH81Y remained unaffected by changes in pH, indicating that of the four His residues present in the HLH-LZ of Max, only protonation of His^{81} played a role in destabilizing the homodimer. Its accessibility to solvent indicated that this re-

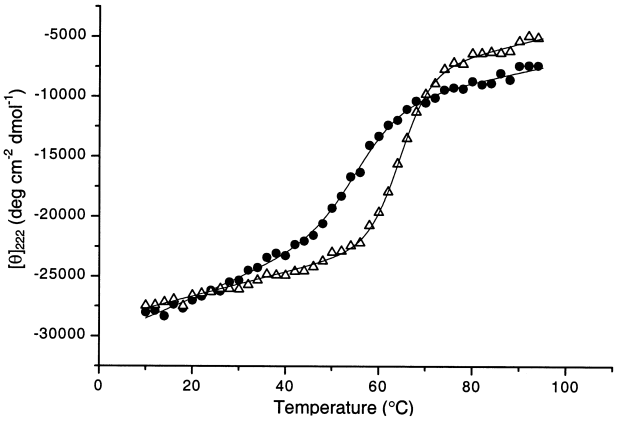

Fig. 3. Thermal denaturation curves of MaxN (●) and MaxN78V (△). The T_m values of MaxN and MaxN78V were 56 and 65°C, respectively.

gion of the LZ might possess a degree of flexibility or laxity that allows entry to solvent molecules. Alternatively, His^{81} may be solvent accessible during brief periods of unfolding and refolding that are present in the dynamic dimer–monomer equilibrium.

The biological significance of His^{81} solvent accessibility is open to question. It is unlikely that His^{81} is predominantly charged in vivo, as further destabilization of the homodimer due to protonation does not occur until below pH 6.8 (Fig. 2). Additionally, studies of MaxN and MaxH81A have indicated that His^{81} is not a target for phosphorylation by histidine kinase (Attwood, P., Tchan, M. and Weiss, A.S., unpublished observations). It may be that the observed solvent accessibility does not reflect a further function for this unusual residue, rather it merely indicates that the His sidechain enables the separation of homodimer strands.

Asn^{78} was also shown to destabilize the Max homodimer. Replacement of this residue with a Val resulted in the thermal stability increasing from 56 to 65°C. This is consistent with results described for similar mutations in the GCN4 and Jun homodimers [26,27], although the degree of stabilization observed in MaxN78V is not as significant as that seen in the GCN4 and Jun mutants, which showed increases in T_m of 42 and 23°C, respectively.

These results indicate that, in contrast to Asn^{92}, Asn^{78} and His^{81} destabilize the Max HLH-LZ homodimer. As Max is the essential binding partner for both the Myc and Mad families of transcriptions factors, it is vital that Max is able to go from a homodimer to the various heterodimer forms rapidly in response to varying levels of its dimerization partners. The placement of Asn^{78} and His^{81} as consecutive and destabilizing *a* and *d* position residues in the center of the HLH-LZ dimerization domain makes them potential candidates for residues important in assisting initiation of the dimer exchange process.

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