Interfacial Asparagine Residues within an Amide Tetrad Contribute to Max Helix-Loop-Helix Leucine Zipper Homodimer Stability*

Received for publication, May 18, 2000, and in revised form, September 5, 2000
Published, JBC Papers in Press, September 7, 2000, DOI 10.1074/jbc.M004264200

Michel C. Tchan‡, Katherine J. Choy‡, Joel P. Mackay‡, Alison T. L. Lyons‡, Naresh P. S. Bains§, and Anthony S. Weiss‡¶
From the ‡Department of Biochemistry, University of Sydney, Sydney, New South Wales 2006, Australia and the §Department of Biochemistry, University of Connecticut Health Center, Farmington, Connecticut 06030

The transcription factor Max is the obligate dimerization partner of the Myc oncoprotein. The pivotal role of Max within the Myc regulatory network is dependent upon its ability to dimerize via the helix-loop-helix leucine zipper domain. The Max homodimer contains a tetrad of polar residues at the interface of the leucine zipper domain. A conserved interfacial Asn residue at an equivalent position in two other leucine zipper proteins has been shown to decrease homodimer stability. The unusual arrangement of this Gln-Asn/Gln-Asn tetrad prompted us to investigate whether Asn92 plays a similar role in destabilizing the Max homodimer. This residue was sequentially replaced with aliphatic and charged residues. Thermal denaturation, redox time course and analytical ultracentrifugation studies show that the N92V mutation does not increase homodimer stability. Replacing this residue with negatively charged side chains in N92D and N92E destabilizes the mutant homodimer. Further replacement of Gln91 indicated that H bonding between Gln91 and Asn92 residues is not significant to the stability of the native protein. These data collectively demonstrate the central role of Asn92 in homodimer interactions. Molecular modelling studies illustrate the favorable packing of the native Asn residue at the dimer interface compared with that of the mutant Max peptides.

The human Max protein was identified by Blackwood and Eisenman (1) as the dimerization partner of the Myc oncoprotein. Heterodimerization with Max has been shown to be essential for the DNA binding, transcription activating, transformative, and apoptotic properties of Myc (2). Max forms homodimers in addition to heterodimers with other members of the basic helix-loop-helix (HLH) leucine zipper (LZ) family of transcription factors. These include Mxi1 (3); Mad1, Mad3, and Mad4 (4, 5); and Mnt (6, 7). Max has also been shown to associate with a member of the DNA-binding domain family of transcription factors, TFE-1 (8). However, of the proteins involved in this complex regulatory network, only Max and Mnt form homodimers under physiological conditions. The role of the Max-associated bHLH LZ proteins in the regulation of cell growth, differentiation, and apoptosis depends upon dimerization with Max (9, 10).

The HLH family of proteins is characterized by a domain consisting of two amphipathic α-helices joined by a short, non-conserved loop of random coil. HLH dimers form a globular fold of four interacting α-helices (11, 12) in which stability and specificity are mediated through highly conserved hydrophobic residues at the core of the HLH domain (13).

The leucine zipper is a dimerization motif characterized by a heptad repeat of leucine residues (14, 15). Folded dimers form a parallel, coiled-coil of α-helices in which, by conventional nomenclature, (abcedfg), the heptad repeat Leu residues occupy position d at the dimer interface. Leu is highly conserved at this position throughout the LZ family of proteins, presumably because its side chain size and structure is such that it provides optimal hydrophobic packing. Another heptad repeat of hydrophobic residues, typically the β-branched amino acids such as Ile and Val, are found in position a at the dimer interface. There is debate over the role of interhelical salt bridges between charged residues in positions e and g in the coiled-coil. Unfavorable e-g electrostatic interactions, where an apostrophe indicates a residue from the opposing chain in the dimer, have been shown to inhibit dimer formation (16) and potentially promote strand exchange. However, the presence of attractive e-g ion pairs may not contribute significantly to dimer stability; indeed, they may be less stabilizing than an equivalent neutral charge interaction (17).

The HLH LZ dimer interacts as a parallel, left-handed, four-helix bundle, with each monomer consisting of two right-handed α-helices joined by the loop region of the HLH domain. The LZ extends as a coiled-coil out of the globular fold formed by the HLH domain. Crystal structures of two HLH LZ proteins, USF and Max, show that the dimerization domain is a single, contiguous functional element, with helix-2 of the HLH extending into the LZ such that there is no clear demarcation between the two domains (18–20). Horiiuchi et al. (21) 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 (20, 22).

The majority of LZ proteins contain 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 (23). NMR studies of a recombinant Jun LZ domain indicate this fact.

© 2000 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.
peptide showed that its interfacial Asn residue is hydrogen bonded (H bonded) and adopts two distinct, rapidly exchanging conformations in solution (24). Mutation of this Asn to a Leu residue caused changes in dimer stability and oligomerization status comparable with the behavior of the Asn to Val mutant of GCN4. Max contains two such a position Asn residues, Asn^91 and Asn^92. These residues may play a destabilizing role akin to that of the interfacing Asn in Jun and GCN4.

In addition to the classical hydrophobic interactions at the dimer interface, the LZ domain of the Max homodimer exhibits unusual structural features (18, 20). Two regions in particular are unique among the known LZ proteins: an interfacial His-His’ packing at position 81, and a tetrad between Gln-Asn/ Gln’-Asn’ at positions 91 and 92 toward the C terminus of the LZ. These residues are likely to be responsible for the propensity of Max to form a variety of heterodimers rather than Max-Max homodimers.

Studies using isolated LZ domains (25, 26) have analyzed the role of these two regions in the context of the Myc-Max heterodimer. These indicate that a buried salt bridge between His81 of the Max LZ and two opposing glutamate residues of the Myc LZ resolves the problem of having these destabilizing hydrophilic residues at the dimer interface. The proton NMR structure of the Myc-Max LZ heterodimer (27) shows Asn^92 of Max forming an H bond with the backbone carbonyl of Leu^420 of the Myc LZ, thus allowing the burial of the polar residue within the hydrophobic core of the LZ.

Our study sheds light on the function and importance of Asn^92 in the context of the Max dimerization domain. The position of this residue at the dimer interface makes it a likely candidate for one of the destabilizing residues important in facilitating peptide exchange between members of the bHLH LZ regulatory network. There is the potential for an array of H bonds to be formed between the Gln-Asn/Gln’-Asn’ tetrad of residues, in contrast to the relatively simple Asn/Asn’ interaction seen previously in the Jun and GCN4 homodimers. This unusual arrangement makes it an attractive target for mutagenesis studies, with the goal of dissecting the role of Asn^92 in Max HLH LZ homodimerization.

**EXPERIMENTAL PROCEDURES**

**Cloning and Mutagenesis**—A synthetic gene was designed which encoded the HLH and LZ domains of the human Max protein (amino acids 37–105)^2 with an additional Gly-Gly-Gly-Cys at the C terminus. BamHI sites were incorporated onto the 5’ and 3’ ends of the coding sequence to permit insertion into the pGEX-2T expression vector (28). The final DNA sequence was optimized for bacterial expression by replacing rare codons with those by expression screening, and its identity was confirmed by automated DNA sequencing in both strands.

Mutagenic polymerase chain reaction (30) was used to introduce changes into pGEXMax-G+G, yielding the aspartate the pGEXMaxV, glutamate (pGEXMaxE), and valine (pGEXMaxV) mutant clones used in this study. Polymerase chain reaction primers were designed to remove the Gly-Gly-Gly encoding from the 5’ region of HLH LZ sequences. Polymerase chain reaction was also used to create an additional clone encoding the native Max HLH LZ sequence without the N-terminal Poly (pGEXMaxN). The four mutagenic primers were: 1) GGATCCGACCATCAAGAGCTCTTTC, 2) CTGAAAGCAGACA(G/C/ A)CTGCTCATGGGA, 3) TTGGGTCAGAGGAGTCCTTAGG, and 4) GATTTTGAATATGTATCTTTAGCAGCA. Four additional mutagenic primers were used to make the two Q91A mutant forms: 5) CAGACGTTTGAGCTGTTTTC, 6) CGGAATCCTGCTTACGCTT, 7) CAGGACGACGACGACGACG, and 8) CTGAAAGCAGCATGCTGGT-GCTTCG. Expression screening isolated mutant clones and automated DNA sequencing of both strands confirmed their identities.

**Protein Production**—Each GST-Max fusion protein was overexpressed in E. coli DH5α that also contained pBS536. pBS536 contains the 2.2-kb GroEL + EcoRI-HindIII fragment of pOF39 (MGG 202, 435–445) inserted at the EcoRV site within the tet gene of pACYC184 in an orientation that does not permit its expression from the tet promoter. Coexpression of GroEL and GroES from pBS536 increased the amount of soluble GST-Max proteins produced to approximately 50% of total expressed fusion protein (data not shown). Cells were induced with 0.5 ms isopropyl-1-thio-β-D-galactopyranoside and grown at 37 °C for 3 h prior to harvesting. GST-Max fusion proteins were separated from cellular proteins by glutathione-agarose affinity chromatography, and Max proteins were released from the fusion protein at the cleaved thrombin 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) trifluoroacetic acid, 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 ESM (Australian Government Analytical Laboratories).

**CD Measurements**—Each homodimer was diluted from stock solutions into physiological ionic salt buffer (50 mM Tris·HCl, 125 mM NaCl, 1 mM EDTA, pH 7.4) to a final peptide concentration of 0.1 mg/ml. Stock peptide concentrations were determined by UV absorbance at 280 nm (A280) using the extinction coefficient value of 2820 cm⁻¹·M⁻¹ for each Max peptide. Far ultraviolet CD spectra from 200 to 260 nm were collected on a Jasco J-720 spectropolarimeter flushed continuously with N₂ and routinely calibrated with n(+)-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. Additional thermal denaturation data were collected for MaxV over a number of peptide concentrations: 1.0, 0.05, 0.01, and 0.005 mg/ml. Base-line spectra of physiological ionic salt buffer were collected and subtracted from all data prior to conversion into mean residue weight ellipticity values using the following formula.

\[
[\theta]_{222} = \frac{\theta \cdot 100 \cdot MRW}{c \cdot d} 
\]

where mean residue weight ellipticity ([θ]) is expressed in deg cm²·dmol⁻¹, θ is raw ellipticity values, 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 centimetres through the optical cell (26). Estimates of α-helical content were generated by the K2D program (31, 32). The thermal denaturation profile was fitted to either sigmoidal function (i) for MaxN and MaxE or (ii) in the case of MaxV. The thermal denaturation profile of MaxD was not subjected to model fitting.

**Analytical Ultracentrifugation**—The self-association of each Max protein was investigated by sedimentation equilibrium methods on a Beckman XL-A analytical ultracentrifuge. Experiments were carried out at loading concentrations of 150, 125, 41.5, and 13.2 μM, using an An-60ti rotor spinning at 20,000, and 25,000 rpm and at a temperature of 25 °C. Samples were made up as solutions of the purified Max peptide in physiological ionic salt buffer. Sedimentation equilibrium data were collected in double sector cells as absorbance versus radius scans (0.001-cm increments, 10 scans). Scans were collected at 4-h intervals and compared with determine when the samples had reached equilibrium. Analysis of the data was carried out using the NONLIN software (33), and the final parameters were determined using a multivariate nonlinear fitting of all data using either a single species (disulfide-bonded dimer) or a dimer-tetramer model. The goodness of fit was determined by examination of the residuals from the fits and consideration of the χ² values. Partial specific volumes were calculated from the amino acid sequence, and the

---

^2 The amino acid sequence of this protein corresponds to Swiss-Prot number P25912 (1).

---

^3 B. P. Surin and N. E. Dixon, unpublished results.
solvent density was calculated to be 1.00482 g ml$^{-1}$; both calculations were carried out using the program SEDNTERP (34).

**Redox Time Course Experiments**—Owing to its additional Gly residues, Max$^{+}$G was used in place of MaxN for redox experiments to facilitate identification of each protein species. Approximately equimolar amounts of each disulfide-linked HLH LZ homodimer were incubated in redox buffer containing 50 mM Tris-HCl, 125 mM NaCl, 1 mM EDTA, 250 $\mu$M glutathione (oxidized), 250 $\mu$M glutathione (reduced), pH 8.3. Dissolved O$_2$ was removed from the reaction mixture by 10 min of continuous N$_2$ infusion. The reaction mixture was incubated at 37 °C under N$_2$ in an air-tight tube. 100-$\mu$l samples were taken at 0, 3, and 16 h, and a 300-$\mu$l sample was taken after 4 days. Samples were analyzed by HPLC on an analytical Delta Pak C-18 column (Waters, Milford, MA) with a linear gradient identical to that used for peptide purification. Peaks were collected and lyophilized, and their identities were confirmed by ESMS.

The calculated molecular weights for each species are as follows: Max$^{+}$G homodimer (17,293), Max$^{+}$G-glutathione conjugate (8,953), MaxD homodimer (16,992), MaxD-glutathione conjugate (8,783), MaxE homodimer (16,991), MaxE-glutathione conjugate (8,797), MaxV homodimer (16,918), and MaxV-glutathione conjugate (8,766).

**Model Building**—Molecular models were created on a Silicon Graphics workstation using the InsightII suite of programs (version 97.0, Molecular Simulations Inc., 1997). The crystal structure of the HLH LZ domains of the Max homodimer bound to its cognate DNA fragment (18) was used as a template. Hydrogen atoms were added to the crystal structure at standard chemical positions. Two residues were replaced; these were Ala residues present in the pdb coordinates but not present in the originally published human Max sequence (1). The two Ala residues in each monomer were replaced by His$^{92}$ and Gln$^{92}$. Asn$^{92}$ was replaced by either Asp, Glu, or Val residues to create the three mutant model homodimers-MaxD, MaxE, and MaxV. The bound DNA fragment was removed from the system prior to energy minimization calculations. Each model was immersed in a 40 × 115 × 40 Å box filled with water molecules. The potential energy of the molecular system was minimized using the consistent valence force field (35). Energy minimization of the homodimer models was performed as a stepwise process designed to gradually release the molecule from restraints tethering it to Max crystal structure coordinates (36). Hydrogen atoms were relaxed using Steepest Descents for 20 iterations; all other atoms were tethered to their original positions. Peptide side chain atoms and oxygen atoms of water molecules were then released from tethering restraints and energy minimized for a further 20 iterations using the same algorithm. Restraints were released from the rest of the molecule for the final energy minimization of 300 iterations using conjugate gradients. The stereochemical quality of each model was assessed using the program Procheck (37).

**RESULTS**

**CD Measurements**—The CD spectra at 10 °C for each of the peptides is shown in Fig. 1A. $\alpha$-Helicity estimates were 91% for the native protein (MaxN); and 20, 21, and 76% for the mutant proteins, N92$\rightarrow$D (MaxD), N92$\rightarrow$E (MaxE), and N92$\rightarrow$V (MaxV), respectively.

Thermal denaturation of the disulfide bridged homodimers was followed by measurements of $[\theta]_{222}$ from 20 to 95 °C (Fig. 1B). Decreases in absolute $[\theta]_{222}$ correlate with decreasing $\alpha$-helicity and the formation of monomeric peptides from the initial homodimers. Each protein underwent a decrease in helicity and a shift to an increasingly random structure as it was heated. However, the initial helicities and unfolding pathways differed for each of the peptides studied.

MaxN shows a cooperative unfolding event with a melting temperature ($T_m$) of 57 °C. $T_m$ is defined as the temperature at which the half peptide population is folded. The thermal denaturation profile exhibited good correlation to the theoretical curve.

MaxE also displayed a thermal denaturation profile in keeping with a cooperative mechanism of unfolding. However, the exchange of the polar Asn residue for the more bulky, charged side chain of Glu had a strong destabilizing effect. At 10 °C, MaxE showed less ellipticity than MaxN, indicating that the population of MaxE molecules contained less helical secondary structure. The $T_m$ of MaxE was 37 °C, considerably lower than that of MaxN.

MaxV exhibited an unfolding pattern similar to that of MaxN up to approximately 85 °C (Fig. 1B). From 85 to 95 °C, MaxV showed the beginning of a second unfolding transition (Fig. 1C). This second transition was reproducible from peptide concentrations of 0.005–1.0 mg/ml and throughout the series of 22 separate thermal denaturation experiments performed on MaxV. Although the helicity of MaxV at 10 °C was not as marked as that of the native peptide, the Val mutant undergoes an unfolding event at approximately the same temperature, 58 °C compared with 57 °C for MaxN. The second transition had an approximate $T_m$ of 90 °C, although this is only an estimate because the ellipticity values had not plateaued by 95 °C, which is the upper limit of the CD spectrometer.

Additional thermal denaturation experiments performed with MaxV in physiological ionic salt plus 4 $\text{m}$ urea failed to
clarify the characteristics of this second unfolding event (data not shown).

The second transition displayed by MaxV contrasts significantly with the profiles exhibited by the other peptides. For this reason, we wished to exclude the possibility of higher order interactions. The $T_m$ for the first denaturation transition of MaxV was concentration independent across the range of samples studied (Fig. 2A). Data from sedimentation equilibrium measurements carried out on MaxV fitted well to a single species model where the only species present was the disulfide-bonded MaxV dimer with an apparent molecular weight of 18300 ± 1700 Da (Fig. 2B). Fitting the data to a monomer-dimer equilibrium (disulfide bridged dimer-tetramer) gave a marginally better fit, with a dimerization constant of 420 M$^{-1}$. However, under these conditions the protein would be 99% dimeric at 0.1 mg/ml. We propose that both models fitted the data similarly because although the propensity of MaxV to form higher order aggregates appears to be real, under our...
experimental conditions it is small enough that a single species
dimeric model describes the interaction adequately. Taken to-
gether, these results indicate that MaxV is present predomin-
antly as a disulfide bridged dimer under the conditions of this
study.

MaxD was predominantly unfolded for the duration of the
thermal denaturation experiment (Fig. 1B). The unfolding
pathway suggests multiple points of slight inflection, indicat-
ing that the small amount of helical MaxD present melts in a
sequential manner consistent with subdomains unfolding
within the HLH LZ. The melting temperatures of these inflec-
tion points were not determined because it is apparent that
MaxD does not display a dimer to monomer unfolding pattern
under these conditions.

To exclude the possibility that the C-terminal disulfide bond
was artificially imparting the correct dimer alignment and thus
masking any role of the Asn residue in orientating the mole-
cules, the thermal denaturation profiles of MaxN and MaxV were
examined under the reducing conditions imparted by a
5-fold excess of Tris(2-carboxyethyl)phosphine hydrochloride
(TCEP). Neither denaturation profile varied significantly from
those of their respective disulfide bridged homodimers (data
not shown).

Two additional mutant proteins were made to assess
whether Gln91 influenced the stability of the native or Val
mutant proteins. These were MaxAN and MaxAV, in which
Gln91 was replaced by Ala in MaxN and MaxV, respectively.
Thermal denaturation of MaxAN and MaxAV was followed by
measurements of [θ]_222 from 10 to 95 °C (Fig. 3). Both mole-
cules showed unfolding profiles similar to those of MaxN and
MaxV. MaxAN had a T_m of 57 °C, which compared closely to
that of MaxN (58 °C). MaxAV similarly unfolded at a compa-
rollable temperature to that of MaxV (57 and 58 °C, respectively).

Redox Time Course Experiments—To determine whether
there was preferential formation of homodimer or heterodimer
species between mutant Max forms and the native Max se-
quence, dimerization behavior was studied under redox condi-
tions. By virtue of additional Gly residues at the N terminus of
the native sequence, Max1G was used in place of MaxN for
redox experiments. This facilitated identification of each pro-
tein species. In each case, the native peptide showed a weak
propensity to form mixed disulfides with glutathione from an
initially homodimeric state. After 4 days, 5–14% of Max1G was
covalently bound to glutathione.

MaxD showed a strong preference to dissociate from an ini-
tially homodimeric form into a mixed disulfide with glutathione
(MaxD-SG). The MaxD-SG form was evident after only 3 h.
After 4 days, 46% of the MaxD peptide was present as a
mixed disulfide with glutathione, however, to a lesser degree
than MaxD; after 4 days, only 5% of the Max+G peptide was
present in this form. Max+G showed no propensity to interact
with MaxD.

MaxE behaved similarly to MaxD. It showed the presence of
a small amount of glutathione adduct after 3 h, and after 4 days
this had increased to 68% of the MaxE peptide (Fig. 5). Max+G
again displayed a weak propensity to dissociate from its ho-
modimeric form and react with glutathione. After 4 days, 14%
of the MaxG peptide was covalently bound to glutathione. ESMS analysis of the minor peak 4 identified two contributing species, suggesting a mixture of dimer components.

The MaxV mutant displayed a similar monomer-dimer distribution to that of the native MaxG protein. After 4 days, 9% of the MaxG peptide and 5% of the MaxV peptide had formed mixed glutathione disulfides (Fig. 6). There was no evidence of interaction between the MaxG and MaxV peptides.

Molecular Modelling—Computer models of the native Max homodimer and the various mutant homodimers were constructed to gain an understanding of interactions at the dimer interface, particularly those occurring at the tetrad of Asn-Gln91-Asn92-Gln91 residues.

The model MaxN homodimer was based on the truncated Max bHLH LZ crystal structure and shows Gln91 and Asn92 in close apposition (Fig. 7A). These residues participate in a tetrad with the Gln91 and Asn92 side chains of the opposing monomer. The burial of these four polar residues at the dimer interface is a substantially different conformation to that of the classical Asn/Asn91H bonding seen in other LZ peptides (1). All four side chains are in positions where they may form H bonds.

The model of the MaxV homodimer shows the hydrophobic side chain of each Val residue directed toward its Val counterpart at the dimer interface (Fig. 7C). This occurs at the expense of the burial of the Gln side chains, which, in comparison to their positions in the native homodimer, are displaced outwards from the dimer interface. The close apposition of the Val side chains provides a continuation of the hydrophobic LZ interface.

The Asp side chains of MaxD are positioned close to the amide protons of the opposing Gln residues (Fig. 7B). However, charge repulsion between the Asp residues on different strands is likely to counteract the benefits of any attractive forces generated between Asp and Gln. Presumably, it is this repulsion that results in a cavity at the dimer interface.

Molecular modelling of the MaxE homodimer reveals Glu side chains forming intramolecular salt bridges with Lys89 (Fig. 7D). There is no evidence for intermolecular interactions at this level of the LZ; indeed there is considerable space between the monomers, presumably caused by repulsion between the negatively charged Glu side chains.

DISCUSSION

Previous studies on interfacial Asn residues have indicated that they play an important role in homodimer destabilization, facilitating zipper exchange, and promoting heterodimer formation. The novel nature of the Asn-Gln/Asn91-Gln91 tetrad affords an opportunity to extend our knowledge of the role of these polar residues.

CD spectra analysis revealed MaxN to be the most helical of the peptides studied. The estimation of 91% $\alpha$-helicity for MaxN at 10 °C indicates that approximately 66 of 74 possible residues are in an $\alpha$-helical conformation. This correlates with the work of Horiuchi et al. (21), who reported that 65 residues...
Gln residues are shaded dark gray, Asn, Val, Asp, and Glu residues are shaded light gray in each model, respectively. Lys is displayed as light gray rendering in the MaxE homodimer model.

Asn residue does not increase the stability of the MaxV homodimer over that of the native MaxN peptide (Figs. 1B and 6). The MaxN homodimer was estimated to be 15% less helical than MaxN at 10 °C (Fig. 1). This is in contrast to the effect of similar hydrophobic substitutions in other LZ peptides, which were shown to dramatically increase the stability of the mutant homodimers. The corresponding Asn to Leu mutant of Jun undergoes a thermal denaturation transition corresponding to a dimer to monomer unfolding event at 75 °C, in comparison with 52 °C for the native peptide (24). GCN4 increased its Tm from 53 to 95 °C upon incorporating a Val residue in place of the naturally occurring a position Asn (23). In contrast, MaxV and MaxN undergo unfolding transitions at 58 and 57 °C, respectively. Both peptides also displayed a similar tendency to remain as homodimers under redox conditions.

The possibility that the 58 °C transition event of MaxV represented an oligomer to dimer unfolding event, thus pointing to the second denaturation (at >90 °C) as the true dimer to monomer melting temperature, was excluded by varying the peptide concentration of MaxV during thermal denaturation studies. Both unfolding events were shown to be concentration-independent (Fig. 2A) and hence represent partial unfolding of the disulfide bridged dimer rather than the melting of associating MaxV dimers. Sedimentation equilibrium studies support this conclusion (Fig. 2B), indicating that less than 1% of the total protein present could be in a tetrameric form at 0.1 mg/ml. Because each MaxV dimer has two contiguous domains containing four α-helices, it is likely that the entire molecule does not unfold cooperatively in a simple two-state manner. The data indicate a stepwise model, with the majority of the protein melting at 58 °C and a residual region unfolding at around 90 °C.

MaxD and MaxE displayed considerably less secondary structure at 10 °C than either MaxV or MaxN (Fig. 1A), as well as being considerably less stable as dimers in the redox time course assay (Figs. 4 and 5). Thus, it is unlikely that the unfolding data reflect a cooperative unfolding of dimer and tetramer species in the two charged mutants. Sedimentation equilibrium studies supported these results by confirming that both MaxD and MaxE were predominantly (>90%) disulfide bridged dimers under the conditions of this study (data not shown).

The close proximity of the polar side chains of Gln to the Val residues of MaxV may have had a negative influence on any stabilizing effects of this mutation, thus masking the true effect of the N92V substitution. Thermal denaturation studies of MaxAV showed that this was not the case (Fig. 3). We conclude that replacement of Asn with a Val residue does not increase the stability of the native protein.

Because the crystal structures of Max were determined at 2.8 and 2.9 Å, it is not possible to identify the H bonding patterns of the amide tetrad residues. Additionally, in the full-length structure the LZ is disordered beyond Asn; hence the exact positions of the tetrad side chains cannot confidently be assigned. Inspection of the MaxN model structure revealed the possibility for multiple, albeit nonideal, H bonding arrangements at the level of the Asn-Gln/Asn-Gln tetrad (Fig. 7A). However, the similar Tm values of MaxAN and MaxN (Fig. 3A) showed that H bonding between Gln and Asn residues does not contribute significantly to the stability of the native protein.

We propose a model of interaction where the relative stability of MaxN may be accounted for by H bonds formed across the dimer interface between the symmetrically oriented Asn side chains, because these residues are positioned differently to the equivalent asymmetrical polar elements of GCN4 and Jun. It is unlikely that Asn is involved in a conformational exchange process comparable with that of the interfacial Asn residues in the Jun LZ (24), because the truncated Max crystal structure indicates that both Asn residues are in a symmetrical conformation. Additionally, motional averaging by these Asn residues would necessitate the displacement of the Gln side chains from their positions at the dimer interface. Hence, where GCN4 and Jun are destabilized by the movement of asymmetrical Asn residues, Max is not destabilized, possibly because Asn is able to form permanent H bonds between symmetrical side chains.

Remarkably, the presence of four polar residues at the dimer interface is not destabilizing to the MaxN homodimer. The extent of unfavorable desolvation of the polar Asn residues at
the hydrophobic dimer interface is, therefore, not as substantial as occurs in the LZs of Jun and GCN4. We propose that H bonds between the Asn side chains compensate for the presence of these buried polar entities.

REFERENCES

1. Blackwood, E. M., and Eisenman, R. N. (1991) *Science* **251**, 1211–1217
2. Amati, B., Brooks, M. W., Levy, N., Littlewood, T. D., Evan, G. I., and Land, H. (1993) *Cell* **72**, 233–245
3. Zervos, A. S., Gyuris, J., and Brent, R. (1994) *Cell* **72**, 233–232
4. Ayer, D. E., Kretzner, L., and Eisenman, R. N. (1993) *Cell* **72**, 211–222
5. Hurlin, P. J., Queva, C., Koskinen, P. J., Steingrimsson, E., Ayer, D. E., Copeland, N. G., Jenkins, N. A., and Eisenman, R. N. (1995) *EMBO J.* **14**, 5646–5659
6. Meroni, G., Reymond, A., Alcalay, M., Borsani, G., Tanigami, A., Tonlorenzi, R., Nigro, C. L., Messali, S., Zollo, M., Ledbetter, D. H., Brent, R., Ballabio, A., and Carrozzo, R. (1997) *EMBO J.* **16**, 2892–2906
7. Gupta, M. P., Amin, C. S., Gupta, M., Hay, N., and Zak, R. (1997) *Mol. Cell. Biol.* **17**, 3924–3936
8. Bouchard, C., Staller, P., and Eilers, M. (1998) *Trends Cell Biol.* **8**, 202–206
9. Bonfield, C., Staller, P., and Eders, M. (1998) *Trends Cell Biol.* **8**, 202–206
10. Ferekidou, A., Amin, C. S., Gupta, M., Hay, N., and Zak, R. (1997) *Mol. Cell. Biol.* **17**, 3924–3936
11. Wendt, H., Thomas, R. M., and Ellenberger, T. (1998) *J. Biol. Chem.* **273**, 5735–5743
12. Fairman, R., Beran-Steed, R. K., and Handel, T. M. (1997) *EMBO J.* **16**, 2892–2906
13. Landschulz, W. H., Johnson, P. F., and McKnight, S. L. (1988) *Science* **240**, 1759–1764
14. Alber, T. (1992) *Carr. Opin. Gen. Dev.* **2**, 205–210
15. O'Shea, E. K., Klemm, J. D., Kim, P. S., and Alber, T. (1991) *Science* **254**, 539–544
16. Laskowski, R. A., Moss, D. S., and Thornton, J. M. (1993) *J. Mol. Biol.* **231**, 1049–1067
Interfacial Asparagine Residues within an Amide Tetrad Contribute to Max Helix-Loop-Helix Leucine Zipper Homodimer Stability
Michel C. Tchan, Katherine J. Choy, Joel P. Mackay, Alison T. L. Lyons, Naresh P. S. Bains and Anthony S. Weiss

J. Biol. Chem. 2000, 275:37454-37461.
doi: 10.1074/jbc.M004264200 originally published online September 7, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M004264200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 35 references, 8 of which can be accessed free at http://www.jbc.org/content/275/48/37454.full.html#ref-list-1