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Probing the solution structure of the DNA-binding protein Max by a combination of proteolysis and mass spectrometry

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Abstract

A simple biochemical method that combines enzymatic proteolysis and matrix-assisted laser desorption ionization mass spectrometry was developed to probe the solution structure of DNA-binding proteins. The method is based on inferring structural information from determinations of protection against enzymatic proteolysis, as governed by solvent accessibility and protein flexibility. This approach was applied to the study of the transcription factor Max—a member of the basic/helix-loop-helix/zipper family of DNA-binding proteins. In the absence of DNA and at low ionic strengths, Max is rapidly digested by each of six endoproteases selected for the study, results consistent with an open and flexible structure of the protein. At physiological salt levels, the rates of digestion are moderately slowed; this and the patterns of cleavage are consistent with homodimerization of the protein through a predominantly hydrophobic interface. In the presence of Max-specific DNA, the protein becomes dramatically protected against proteolysis, exhibiting up to a 100-fold reduction in cleavage rates. Over a 2-day period, both complete and partial proteolysis of the Max-DNA complex is observed. The partial proteolytic fragmentation patterns reflect a very high degree of protection in the N-terminal and helix-loop-helix regions of the protein, correlating with those expected of a stable dimer bound to DNA at its basic N-terminals. Less protection is seen at the C-terminal where a slow, sequential proteolytic cleavage occurs, correlating to the presence of a leucine zipper. The results also indicate a high affinity of Max for its target DNA that remains high even when the leucine zipper is proteolytically removed. In addition to the study of the helix-loop-helix protein Max, the present method appears well suited for a range of other structural biological applications.

Keywords: basic-helix-loop-helix-zipper; mass spectrometry; matrix-assisted laser desorption/ionization; Max; peptide mapping; protein-DNA interactions; protein structure; proteolysis

In this paper, a simple approach is presented for investigating structural properties of DNA-binding proteins in solution using a combination of proteolysis and mass spectrometry. The approach was applied to the DNA-binding protein Max. Max is a member of the basic/helix-loop-helix/zipper family of transcription factors that are important for the regulation of cell development and proliferation (Blackwood & Eisenman, 1991; Prendergast et al., 1991). The b/HLH/Z proteins, a subgroup of the helix-loop-helix family (Murre et al., 1989; Littlewood & Evan, 1994), share high sequence homologies in regions that allow them to interact with each other as well as to bind DNA (Baxevanis & Vinson, 1993; Ferré-D'Amaré & Burley, 1995). Max was the first HLH protein whose three-dimensional structure was determined (Ferré-D'Amaré et al., 1993). The crystal structure, solved at 2.9 Å by X-ray crystallography, showed that the Max b/HLH/Z domain binds to its cognate DNA as a symmetric homodimer. Subsequently, the structures of three other homodimeric HLH proteins—all bound to their cognate DNA—have been determined (USF, Ferré-D'Amaré et al., 1994; E47, Ellenberger et al., 1994; MyoD, Ma et al., 1994), providing further structural information about these eukaryotic regulatory proteins. In addition to the crystallographic analyses, studies using genetic (i.e., mutational) and biochemical techniques have

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Abbreviations: b/HLH/Z, basic/helix-loop-helix/zipper; FWI, formic acid–water–isopropanol; 4HCCA, 4-hydroxy-α-cyano cinnamic acid; MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry; nsDNA, nonspecific DNA; sDNA, specific DNA; V8, endoprotease Glu-C.

provided insight into structural features that control the dimerization, DNA-binding specificity, and functions of Max and other HLH proteins (reviewed in Littlewood & Evan, 1994).

In vivo, homodimers of Max have been suggested to repress transcriptional activation (Kretzner et al., 1992; Amati et al., 1993). It is known that a heterodimer forms between Max and its in vivo partner, Myc, an important b/HLH/Z proto-oncoprotein. The Myc/Max dimer has been shown to activate transcription and has been implicated in neoplastic cell transformation (Blackwood et al., 1992; Kato et al., 1992). To further elucidate the biochemical mode of action of Max, Myc, and other members of the biologically important helix-loop-helix family, it is necessary to understand the relationship between the structures and functions of these proteins in solution. Although multidimensional NMR spectroscopy and mutational analyses are of pivotal utility for solution studies of proteins, it remains desirable to develop additional complementary biochemical approaches that can be applied with ease and speed.

We present an approach that combines enzymatic proteolysis with mass spectrometry to investigate the solution behavior of DNA-binding proteins. Our method involves performing time-course proteolytic digestions of the protein in both the absence and presence of DNA. Evaluation of the resultant proteolytic fragmentation patterns along with the rates of digestion provides information about cleavage site accessibility and flexibility of the protein in solution. Fundamental to this approach is the notion of protection against proteolysis. Protection is conferred on regions of the protein that are buried, are within a rigid structure, or are involved in protein–protein or protein–DNA interactions. In contrast, regions of the protein that are solvent accessible and flexible or unstructured will be less protected and therefore susceptible to proteolytic cleavage (Fruton, 1975; Fontana et al., 1986; Hubbard et al., 1994). An essential requirement of the method is a facile and accurate identification of the proteolytic products. The newly developed technique of matrix-assisted laser desorption/ionization mass spectrometry serves this analytical need well because of its ability for measuring the molecular masses of mixtures of peptides and proteins (Beavis & Chait, 1990a; Hillenkamp et al., 1991; Chait & Kent, 1992; Aebersold, 1993; Wang & Chait, 1994). The MALDI technique is ideal for direct mapping of protein digests because of its relatively high mass accuracy (0.01–0.05%), rapid measurement time (minutes), and minimal sample-handling requirements (Beavis & Chait, 1990a; Billeci & Stults, 1993; Tsarbopoulos et al., 1994). The principle of the mass spectrometric proteolytic assay is illustrated in Figure 1.

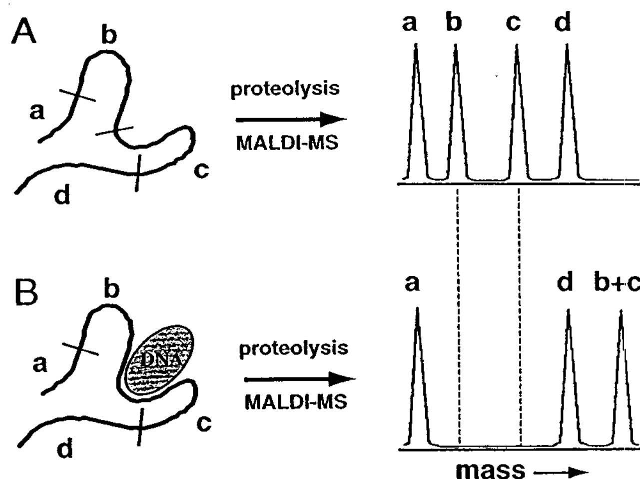

Fig. 1. Proteolytic protection assay. An illustration of the mass spectrometric proteolytic protection assay. The figure compares mass spectrometric peptide maps obtained by enzymatic proteolysis of a hypothetical DNA-binding protein, in the absence and presence of DNA. A thick black line traces the polypeptide backbone of the protein containing three proteolytic cleavage sites. **A:** In the absence of DNA, the protein is proteolytically cleaved into four peptide fragments (a, b, c, and d). The mass spectrum of the resulting digest shows four peaks with masses that can be determined with sufficient accuracy to unambiguously identify the four peptide fragments. **B:** In the presence of DNA, one of the three potential sites of proteolysis becomes protected following DNA binding. Under these conditions, the protein is cleaved into three fragments (a, d, and b + c) that can be identified in the mass spectrum. Because the site between b and c is protected against proteolysis, a single peak, corresponding to the combined fragment b + c, appears at higher mass. Comparison of the mass spectra provides information regarding the DNA-binding region. The scheme illustrated is a simplification of the actual requirements for cleavage. In addition to accessibility, there is also the necessity that the cleavage sites be located within flexible segments of the protein, in order to optimize interaction with the extended active site of the protease (Fruton, 1975; Hubbard et al., 1994).

The Max protein

Full-length, wild-type Max consists of 160 residues (Blackwood & Eisenman, 1991; Prendergast et al., 1991). We studied the b/HLH/Z domain of Max (Ala 22–Thr 113; Fig. 2A). All subsequent references to Max are to the b/HLH/Z form. This truncated form of the protein was used in the original X-ray crystallographic study of the Max–DNA complex (Ferré-D'Amaré et al., 1993). The various structural regions of Max are indicated in a linear representation of the protein (Fig. 2B). The DNA recognition site for high-affinity binding of Max (termed the E-box) consists of the six-base pair palindrome CACGTG. There are four features deduced from the X-ray crystallographic structure of the Max–DNA complex that are of consequence for the present study. These are: (1) Max binds the major groove of E-box DNA as a symmetric homodimer, with its N-terminal α-helical basic regions grabbing the DNA in a "scissors grip" fashion; (2) the HLH region folds into a parallel, left-handed, four-helix bundle that is apposed to the DNA; (3) a parallel coiled coil (i.e., a "leucine zipper") extends immediately C-terminal to the four-helix bundle; and (4) C-terminal to the zipper, the protein becomes random coil.

No X-ray crystallographic information is available on the structure of HLH proteins in the absence of DNA. Little is known about the structure of the free proteins, although NMR spectroscopy has been used to study a disulfide-linked dimer of a MyoD-bHLH peptide, which is inactive in DNA binding (Starovasnik et al., 1992), and electron paramagnetic resonance spectroscopy has been used to distinguish between alternate folding topologies of a MyoD bHLH peptide (Anthony-Cahill et al., 1992). In the present study, we address several questions concerning helix-loop-helix proteins using Max as an example. What are the structural characteristics of Max in the absence of DNA? How does solution ionic strength affect the structure of the protein? How does the presence of DNA alter the protein? How does the solution structure of the Max–DNA complex compare to the structure deduced from X-ray crystallography?

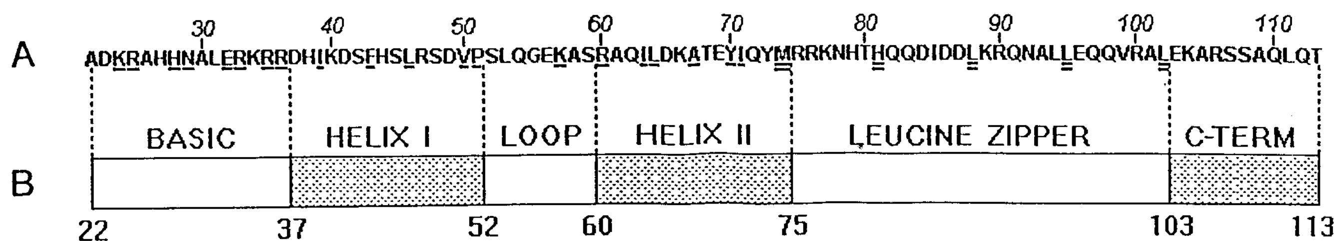

Fig. 2. b/HLH/Z Max. **A:** Amino acid sequence of the b/HLH/Z form of human Max. Amino acid residue numbering (22–113) is based on the full-length protein (160 residues). Residues conserved throughout the b/HLH/Z protein family are singly underlined. Heptad repeat residues of the leucine zipper are doubly underlined. The basic region is the DNA-binding region. Met 74 is a conserved residue of Helix II and is also the first member of the leucine zipper heptad. **B:** Linear diagrammatic representation of b/HLH/Z Max showing the basic, helix I, loop, helix II, leucine zipper, and C-terminal regions.

Results

The proteolytic protection assay outlined in Figure 1 and described in the Materials and methods section forms the basis of the solution study of Max. The protein (with and without DNA) was subjected to time-course measurements of proteolysis employing six different endoproteases: Glu-C (V8), Lys-C, Asp-N, trypsin, chymotrypsin, and subtilisin. MALDI-MS was used to follow the digestions and accurately map the sites of cleavage in the protein.

V8 proteolysis in the absence and presence of Max-specific DNA

Figure 3 compares the time-courses of a V8 digest of Max in the absence and presence of DNA. V8 protease targets primarily glutamate residues, of which Max has five (see Fig. 2 and Fig. 3, bottom). The digestions were performed in 50 mM buffer and at a low salt concentration (15 mM KCl). The masses of the proteolytic fragments were determined with sufficient accuracy to enable their unambiguous identification. In the absence of DNA, V8 protease digests Max very rapidly (Fig. 3A,B,C). After just 2 min, considerable cleavage is observed from all five glutamate residues (Fig. 3B). By 1 h, the digestion is nearly complete (Fig. 3C). These findings demonstrate that, in the absence of DNA and at low salt, Max adopts a form that is highly susceptible to V8 proteolysis, correlating to a rather open and flexible structure. The presence of a 50% excess molar quantity (over protein) of a 14-mer Max-specific DNA (sDNA; see Materials and methods) leads to a dramatic decrease in the proteolytic susceptibility of the protein. Thus, after a 2-min period, Max exhibits no V8 digestion (compare Fig. 3E to Fig. 3B). After 1 h, only a hint of digestion has occurred and this from the C-terminal (fragment 22–103, Fig. 3F). Results of the MALDI-MS peptide mapping after 1 h of digestion are summarized diagrammatically below Figure 3C (without DNA) and Figure 3F (with sDNA). Even following 48 h of digestion, a significant fraction of Max remains undigested (Fig. 4). Addition of fresh protease after this time does not alter the pattern of digestion. The fragmentation that does occur by 48 h falls into two categories. The first resembles the complete digestion observed in the absence of DNA. The second arises from highly selective partial proteolysis of Max to yield two C-terminally truncated fragments, 22–103 and 22–96. These two fragments appear sequentially, first 22–103 (after 1 h, Fig. 3F) followed by 22–96 (after several hours, Fig. 4). The long persistence of intact protein and fragments 22–103 and 22–96 indicates that in the presence of sDNA, Max adopts a form that is highly protected against V8 proteolysis. In addition, fragments 22–96 and 22–103 retain a high level of protection against further digestion, suggesting that these fragments remain tightly bound to DNA. Results of the 48-h *partial* V8 proteolysis are summarized in the diagram at the bottom of Figure 4.

V8 proteolysis in the presence of nsDNA

Figure 5A shows a 5-min V8 digest of Max performed in the presence of a 14-mer nonspecific DNA, which does not contain the E-box Max recognition site (TATA DNA; see Materials and methods). From Figures 5A and 3B, it is seen that the rate of digestion of Max has slowed in the presence of nsDNA compared to that in the absence of DNA. The rate of digestion, however, is considerably greater than that observed in the presence of sDNA (compare Figs. 5A and 3F). Figure 5A shows that after 5 min of digestion of Max in the presence of nsDNA, two principal components dominate. The first is undigested Max and the second is the fragment 33–113, which arises from a cleavage in the N-terminal region of the protein. A minor amount of cleavage from the C-terminal region is also seen (fragments 33–103, 22–96, and 22–103). This pattern of fragmentation shows that, in the presence of nsDNA, susceptibility to V8 proteolysis follows the order N-terminal region $\gg$ leucine zipper $>$ four-helix bundle. Following rapid cleavage of the N-terminal of the protein, digestion of the remaining V8 sites is slower, requiring more than 4 h for completion (data not shown). These results indicate that the presence of nsDNA reduces the susceptibility of the helix-loop-helix-zipper regions of Max to V8 proteolysis, possibly through nonspecific DNA interactions. In an attempt to diminish such nonspecific interactions, a V8 digest of Max (in the presence of nsDNA) was carried out at 150 mM KCl (data not shown). The rates and patterns of V8 cleavage of Max under these conditions were similar to those obtained at 15 mM KCl. It should be recognized, however, that an elevated salt level *by itself* promotes a tightening of the dimeric structure of the protein (see below).

V8 proteolysis and the effects of ionic strength

Increasing the ionic strength had notable effects on the V8 proteolysis of Max in the absence of DNA. Figure 5B shows a 5-min

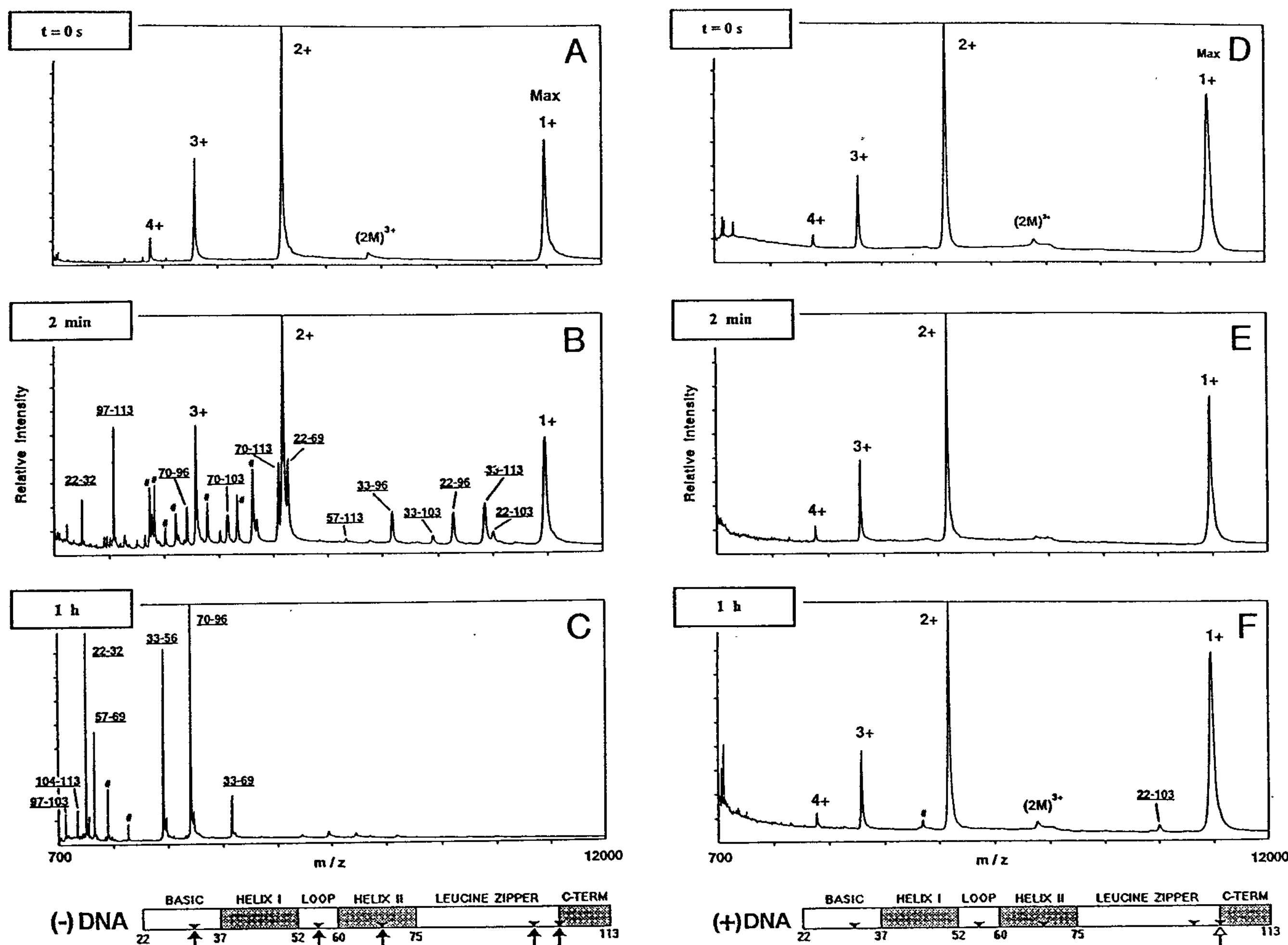

Fig. 3. V8 digestion of Max, (−) and (+) DNA. MALDI mass spectra of the products of a time-course V8 proteolysis of b/HLH/Z Max in the (A, B, C) absence and (D, E, F) presence of Max-specific DNA. Digests were performed in 15 mM KCl at pH 6 buffer and 25 °C (see Materials and methods). Three time points are shown: A, D: 0 s (before the addition of V8); B, E: 2 min of digestion; and C, F: 1 h of digestion. Peaks labeled Max 1+ arise from the singly protonated monomeric Max protein. Peaks labeled 2+, 3+, and 4+ arise from multiply protonated monomeric Max, resulting from the MALDI process. Peak labeled $(2\,M)^{3+}$ also originates from the MALDI process. Peaks corresponding to singly charged V8 fragments are labeled with their sequence as determined from their measured mass (accuracy of 0.02%). For clarity only the singly charged fragment peaks are labeled with sequences; the corresponding multiply charged fragments peaks are labeled with the pound symbol, #. Linear diagrams at the bottom of the figure summarize the progress of proteolysis following 1 h of V8 digestion in the absence (C) and presence (F) of Max-specific DNA. Small solid arrowheads inside the diagrams point to the sites of rapid cleavage by protease that are observed in the absence of DNA. For V8 protease, rapid cleavage occurs at the five Max glutamate residues 32, 56, 69, 96, and 103. Large arrows outside the diagram point to the observed sites of proteolysis determined by MALDI-MS—dark shaded arrows signify a rapid and complete cleavage (minutes to hours); open arrows signify a slow cleavage (hours to days).

V8 digest of Max in 50 mM buffer and 150 mM KCl. Compared to the digestion carried out under low salt conditions (15 mM KCl), the elevated ionic strength leads to a modest reduction in the rates of proteolysis and a large change in the patterns of fragmentation (compare Figs. 5B and 3B). At elevated ionic strength, digestion occurs primarily from the N-terminal and to a lesser extent from the C-terminal regions of Max (Fig. 5B). Even after 1 h of digestion, there remains a portion of high-mass peptides: fragments 33–96, 33–103, and 33–113 (data not shown). These findings indicate that physiological salt levels lead to a considerable protection against proteolysis of the four-helix bundle. Two other points are noteworthy. First, the pattern of proteolytic cleavage of Max, measured at physiological salt levels (Fig. 5B), is remarkably similar to the pattern obtained in the presence of nsDNA and low salt (Fig. 5A). Second, moderate ionic strengths (150 mM KCl) have little effect on either the rates or patterns of proteolysis of Max in the presence of sDNA (data not shown).

Proteolysis of the leucine zipper

A sequential proteolytic removal of residues of the leucine zipper was shown to occur during the V8 digestion of Max (Figs. 3D, E,F, 4). Similar and even more striking effects on the leucine zipper were observed with the use of trypsin and subtilisin. Subtilisin has the broadest specificity of all the endoproteases used

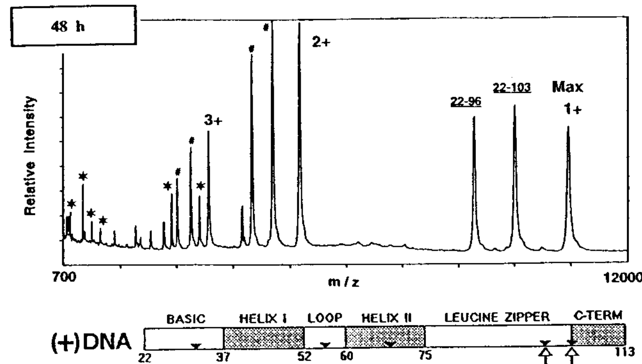

Fig. 4. V8 digestion of Max, (+) DNA at 48 h. MALDI mass spectrum of a 48-h V8 digest of the b/HLH/Z Max-DNA complex. Two peaks, 22–96 and 22–103, are the singly protonated V8 fragments of the protein. Peaks labeled with an asterisk represent complete V8 digest fragments. Linear diagram below the spectrum shows the two V8 cleavage sites of the Max-DNA complex that are accessible to protease (open arrows pointing to Glu 96 and Glu 103). This diagram shows the extent to which Max has undergone proteolysis to fragments that maintain DNA binding. See Figure 3 caption for a full description of all labels and symbols.

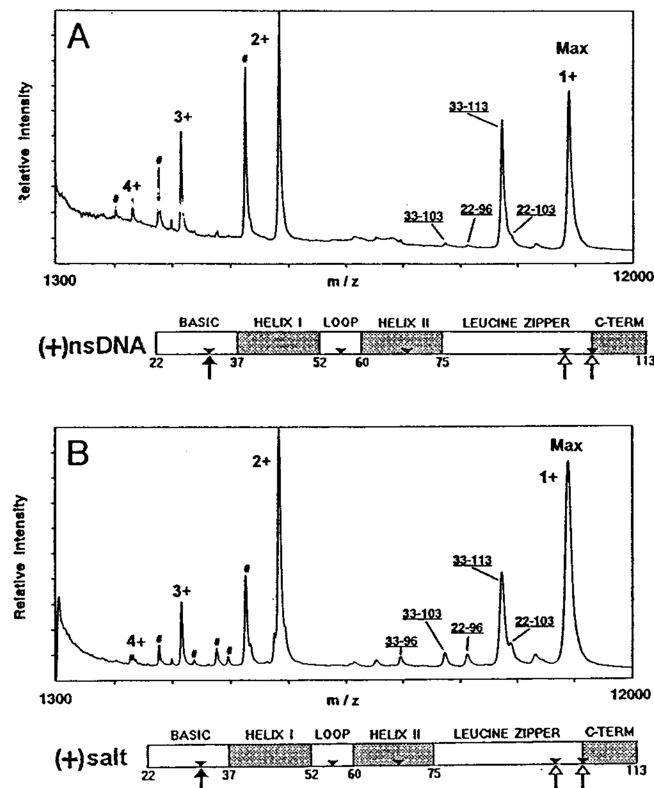

Fig. 5. V8 digestion of Max (+) nsDNA and Max at elevated ionic strength. **A:** MALDI mass spectra of a 5-min V8 digest of b/HLH/Z Max performed in the presence of nsDNA (TATA DNA; see Materials and methods). Digest was carried out at 15 mM KCl. Cleavage at the N-terminal of Max gives rise to the peptide 33–113. **B:** MALDI mass spectrum of a 5-min V8 digest of b/HLH/Z Max in the absence of DNA but at 150 mM KCl. Note similarity to spectrum in A. Diagrams below each spectrum summarize the results. See Figure 3 caption for a full description of all labels and symbols.

in this study. After just 1 min, in the absence of DNA, Max undergoes extensive digestion by subtilisin, leaving very little starting protein intact (Fig. 6A). By 1 h, only small peptide fragments (<2,500 Da) remain (data not shown). This outcome changes dramatically in the presence of sDNA. At 1 min of digestion, a highly selective cleavage has occurred between residues 108 and 109 on a portion of the protein, although a substantial proportion of Max (>50%) remains undigested (Fig. 6B). After 4 h, digestion continues to occur exclusively from the C-terminal region in a sequential manner (Fig. 6C). Over the next 2 days, the fragmentation progresses sequentially through the leucine zipper, in the N-terminal direction until just three fragments dominate: 22–77, 22–83, and 22–84 (Fig. 6D). The progression of cleavage abruptly stalls at residue 77, near the base of the leucine zipper and just above the four-helix bundle. This "unzipping and stalling" pattern also appears in the V8 results (Figs. 3D,E,F, 4) and the trypsin results (data not shown). For the trypsin digestion, the progression of cleavage stalls at residue 75.

Additional proteases

In a manner similar to the above-described experiments with V8, subtilisin, and trypsin, Max was also extensively studied with Endo Asp-N, Lys-C, and chymotrypsin. Several coherent themes emerge by combining the results obtained using all six proteases. First, the cleavage of Max, in the absence of DNA and at low ionic strengths, was virtually complete after 2 h of digestion by any of the six proteases. Elevated ionic strengths slowed the V8 proteolysis considerably (Fig. 5B), but had relatively less effect in slowing the digestions of Max with the five other proteases. A second theme was that, in the presence of sDNA, the rates of digestion of Max were greatly lowered—as previously described for the V8 and subtilisin results. Over a 2-day period, all of the proteases studied showed some degree of complete as well as partial digestion of the Max–DNA complex. The extent of complete digestion varied—V8, Lys-C, and chymotrypsin showed the greatest, and Asp-N showed the least.

The data involving partial digestion are the most informative in characterizing the Max–DNA structure. Results for four of the proteases are summarized in Figure 7. (Similar summaries for V8 and subtilisin are given in Figures 4 and 6, respectively.) The diagrams depicted in these figures show the observed sites of cleavage, which lead to fragments *that maintain DNA binding*. The Lys-C and chymotrypsin results can serve as examples for elucidation. Over a period of 3 days, Lys-C proteolysis of the Max–DNA complex leads to complete as well as partial digestion (data not shown). Partial proteolysis results in three high-mass peptides: 25–77, 25–104, and 25–113. These fragments show significant resistance to digestion, suggesting that they remain bound to DNA. These findings are summarized on the Lys-C diagram of Figure 7: the solid arrow at the N-terminal signifies complete cleavage (within 12 h) at Lys 24; the open arrows at the leucine zipper and C-terminal regions signify incomplete cleavage (after 1 day) at Lys 77 and 104. In contrast, the chymotrypsin digest of the Max–DNA complex did not produce any long-lived partial proteolytic fragments. After 1 day of chymotrypsin treatment of Max, only undigested protein and a modest amount of completely digested Max was observed (data not shown). Thus, no arrows are drawn in the chymotrypsin diagram depicted in Figure 7.

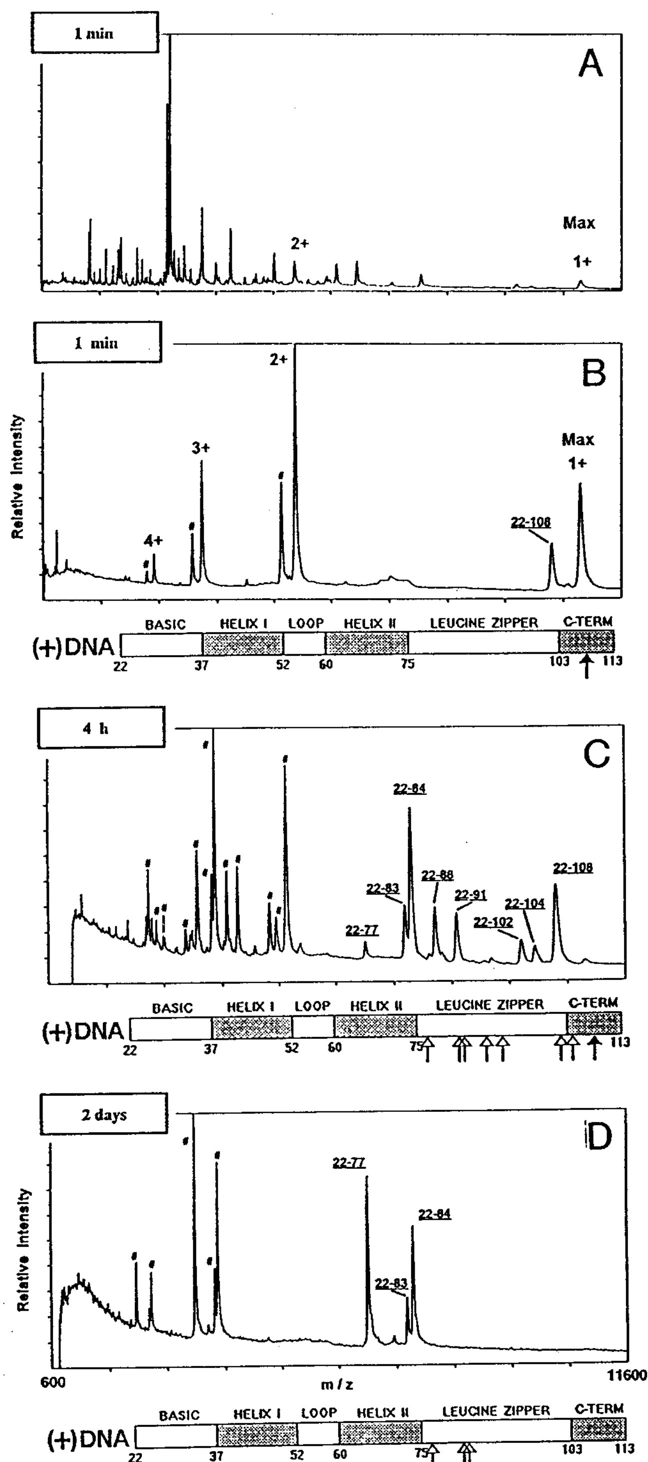

Fig. 6. Subtilisin digest of Max, (−) and (+) DNA. MALDI mass spectra of the subtilisin proteolysis of b/HLH/Z Max. **A:** The 1-min subtilisin digest in the absence of DNA. Extensive cleavage has occurred. The large number of fragment peaks are not labeled. **B, C, D:** The 1-min, 4-h, and 2-day subtilisin proteolysis of Max in the presence of Max-specific DNA. Digests were performed in 15 mM KCl. Diagrams below each panel summarize the progress of proteolysis of Max in the presence of DNA. Rapid cleavage at residue 108 is followed by a slow, sequential cleavage through the leucine zipper. Cleavage stalls at residue 77. See Figure 3 caption for a full description of all labels and symbols.

The proteolytic protection map

We present our current findings in the context of the X-ray crystal structure in Figure 8. Figure 8 shows a ribbon diagram of the b/HLH/Z Max-DNA X-ray crystallographic structure (Ferré-D'Amaré et al., 1993) superimposed with results from our proteolysis experiments. Amino acid residues that are targets of cleavage (by the six endoproteases used in our study) are depicted as colored spheres in a space-filling representation; other residues are not shown (see figure caption for complete description). The colors correspond to the three levels of protection against *partial* proteolysis of the Max-DNA complex that allows for the maintenance of DNA binding. Blue represents the greatest degree of protection, where no partial proteolysis was observed over 2–4 days of digestion. Green indicates a lesser degree of protection, where complete or partial proteolysis at these sites has occurred over this time. Red represents the least amount of protection, where complete cleavage at these residues has occurred in less than 1 day.

Discussion

Max in the absence of DNA

Under conditions of low salt (50 mM buffer, 15 mM KCl, 1 mM $MgCl_2$), Max exhibits little resistance to digestion by six different endoproteases. The rates of digestion of Max are similar to those usually observed for denatured proteins or peptides. With the use of V8 protease, for example, the digestion occurs rapidly and indiscriminately at all five glutamate residues of Max and is nearly complete in 1 h (Fig. 3A,B,C). These findings demonstrate that, at low ionic strengths, Max has an open, flexible structure, one that has insufficient higher-order folding and/or rigidity to resist rapid proteolytic digestion. Although these results do not allow us to conclude that the protein is in an oligomeric form other than a monomer, they suggest that any self-associaion is weak at low ionic strengths.

Elevation of the ionic strength has pronounced effects on the V8 digestion of Max (Fig. 5B), although the effects with the other proteases were less dramatic. These distinctions may be related to the differences in the activities and the modes of action of the proteases. At 150 mM KCl, the rate of V8 proteolysis of Max drops by almost an order of magnitude compared to observations under low ionic strengths. Two factors may account for this reduction of proteolytic rates. Elevated chloride ion has previously been observed to inhibit V8 activity (Sørensen et al., 1991) and is predicted to give an approximate twofold decrease in V8 activity in going from 15 mM to 150 mM KCl. The second factor is based on the notion that elevated ionic strengths may induce a structural change of the protein; evidence for this is suggested by the observed changes in the pattern of cleavage by V8 at elevated salt levels. The cleavage pattern indicates three regions of proteolytic protection that decrease in the order four-helix bundle > leucine zipper ≫ N-terminal region. By extrapolating from the X-ray crystal structure of the Max-DNA complex, this order of proteolytic protection appears to correlate to the extent of hydrophobic interactions found within each region. The greatest number of hydrophobic interactions are found within the four-helix bundle, less in the leucine zipper and fewest in the N-terminal, which, in the absence of DNA, is expected to be a random coil (Ferré-D'Amaré et al., 1994). These corre-

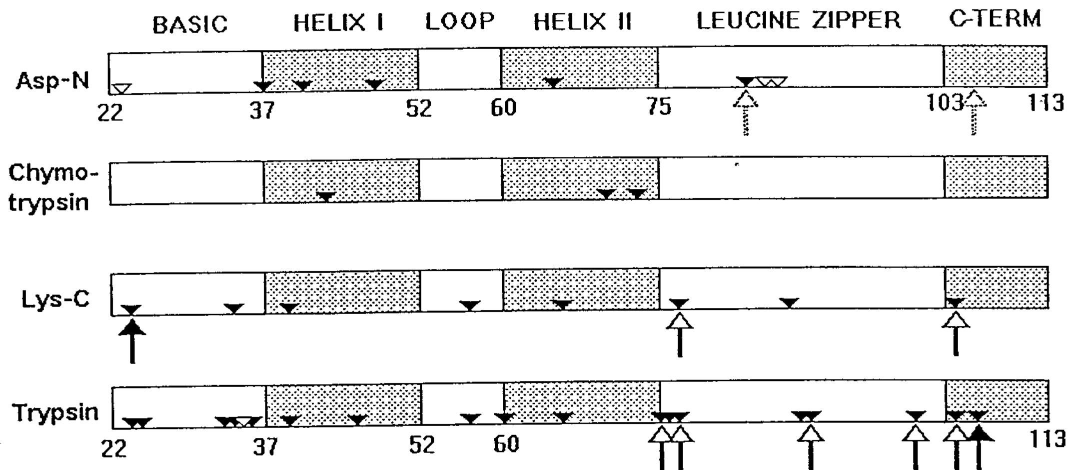

Fig. 7. Partial proteolysis of the b/HLH/Z Max-DNA complex. Results summarizing the proteolysis of b/HLH/Z Max in the presence of DNA by the proteases Asp-N, chymotrypsin, Lys-C, and trypsin. Small arrowheads inside the diagrams point to the respective potential proteolytic cleavage sites. Cleavage sites can be divided into two types: those that are cleaved by protease in the absence of DNA (indicated by the solid arrowheads) and those that are not cleaved in the absence of DNA (indicated by open arrowheads). Large arrows outside the diagrams point to sites of proteolysis of the Max–DNA complex, as measured by MALDI-MS, that result in fragments that maintain DNA binding. Three levels of proteolysis are represented: a solid arrow signifies a rapid and complete cleavage (minutes to hours), an open arrow signifies a slower and nearly complete cleavage (hours to 2 days), and an arrow drawn in broken outline indicates a very slow and incomplete cleavage (2 days or more). Proteolysis by Asp-N included the nonspecific cleavage between Ala 105 and Arg 106, which did not occur in the absence of DNA.

lations of proteolytic protection with hydrophobic interactions indicate that physiological salt levels favor a dimeric form of Max. Because dimerization occurs mainly through hydrophobic interactions found within the four-helix bundle and leucine zipper domains, an elevation of the salt level is predicted to stabilize the dimer.

Max in the presence of DNA

Our findings are summarized as follows. (1) In the presence of DNA, both complete and partial proteolysis of Max were observed, the extent of which was protease dependent. The fragmentation patterns of partial proteolysis were the most informative for our studies. (2) In the presence of DNA, striking changes were observed in the rates and patterns of proteolysis of Max. The effective rates of proteolysis were reduced greatly—by up to a hundred-fold—compared to those seen in the absence of DNA. The steep drop in rates indicates that in the presence of DNA, Max undergoes structural changes, which correlate to a significant reduction of solvent accessibility and/or flexibility of the protein. (3) With each of the proteases used, the patterns of proteolysis were dependent on the nature of DNA present in solution. In the presence of sDNA, the N-terminal of Max remains highly protected against proteolysis, whereas in the presence of nsDNA the N-terminal is rapidly cleaved. This behavior is shown, for example, in the V8 digestion of Max (compare Figs. 3D,E,F and 4 with Fig. 5A). Similar findings are also observed with the Lys-C and trypsin digestions of Max in the presence of nsDNA (data not shown). We infer from our proteolytic protection assay that Max binds at its N-terminal to E-box DNA with high specificity and affinity, strengthening the X-ray crystallographic findings and earlier biochemical studies (Ferré-D'Amaré et al., 1993 and references cited therein). Conversely, in the presence of nsDNA, the N-terminal remains highly susceptible to proteolysis, an indication that it is flexible and accessible to proteolytic attack. This finding is in agreement with CD measurements, which suggest that the N-terminal of b/HLH/Z proteins is a random coil in the presence of nsDNA (Ferré-D'Amaré et al., 1994). (4) The distinction between the patterns of proteolysis of Max in the presence of sDNA and nsDNA supports an "induced fit" model for DNA binding (Spolar & Record, 1994). For Max the induced fit model predicts that site-specific DNA binding would proceed by a random-coil-to-helix folding transition of the N-terminal of the protein. The folding transition occurs only in the presence of DNA containing the cognate E-box recognition site, as evidenced by the rapid cleavage of the N-terminal of Max in the presence of nsDNA. (5) The results of the proteolytic protection assay suggest that the binding of Max to E-box DNA is accompanied by an extensive rigidification of the protein throughout its entire length. Proteolytic susceptibility is greatest only toward the extreme N- and C-termini (summarized in Figs. 7, 8). The MALDI-MS peptide mapping results indicate that there are three distinct regions of proteolytic protection in the presence of Max-specific DNA. These are the N-terminal, the central, and the C-terminal regions.

The N-terminal region

The N-terminal DNA-binding region (residues 24–36) consistently exhibited the greatest protection from proteolysis in the presence of sDNA. This observation is in agreement with the finding that the N-terminal adopts a rigid helical conformation that makes a large number of specific contacts with E-box DNA (Ferré-D'Amaré et al., 1993). The proteolytic protection assay also addresses a specific structural question concerning Lys 24. X-ray crystallography of the Max–DNA complex indicated that