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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.

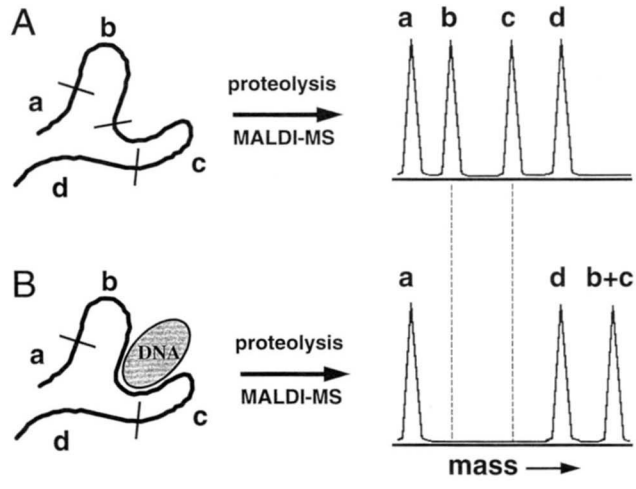

Fig. 1. Proteolytic protection assay. An illustration of the mass spectrometric proteolytic protection assay. The figure compares mass spectrometric peptide maps obtained by enzymatic proteolysis of a hypothetical DNA-binding protein, in the absence and presence of DNA. A thick black line traces the 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?

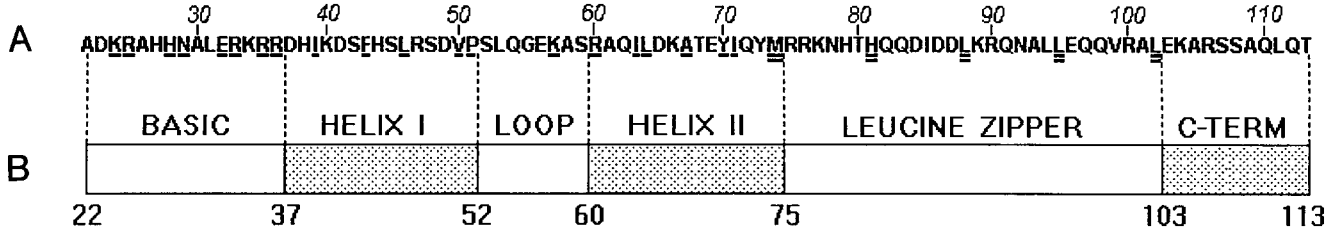

Fig. 2. b/HLH/Z Max. **A:** Amino acid sequence of the b/HLH/Z form of human Max. Amino acid residue numbering (22–113) is based on the full-length protein (160 residues). Residues conserved throughout the b/HLH/Z protein family are singly underlined. Heptad repeat residues of the leucine zipper are 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

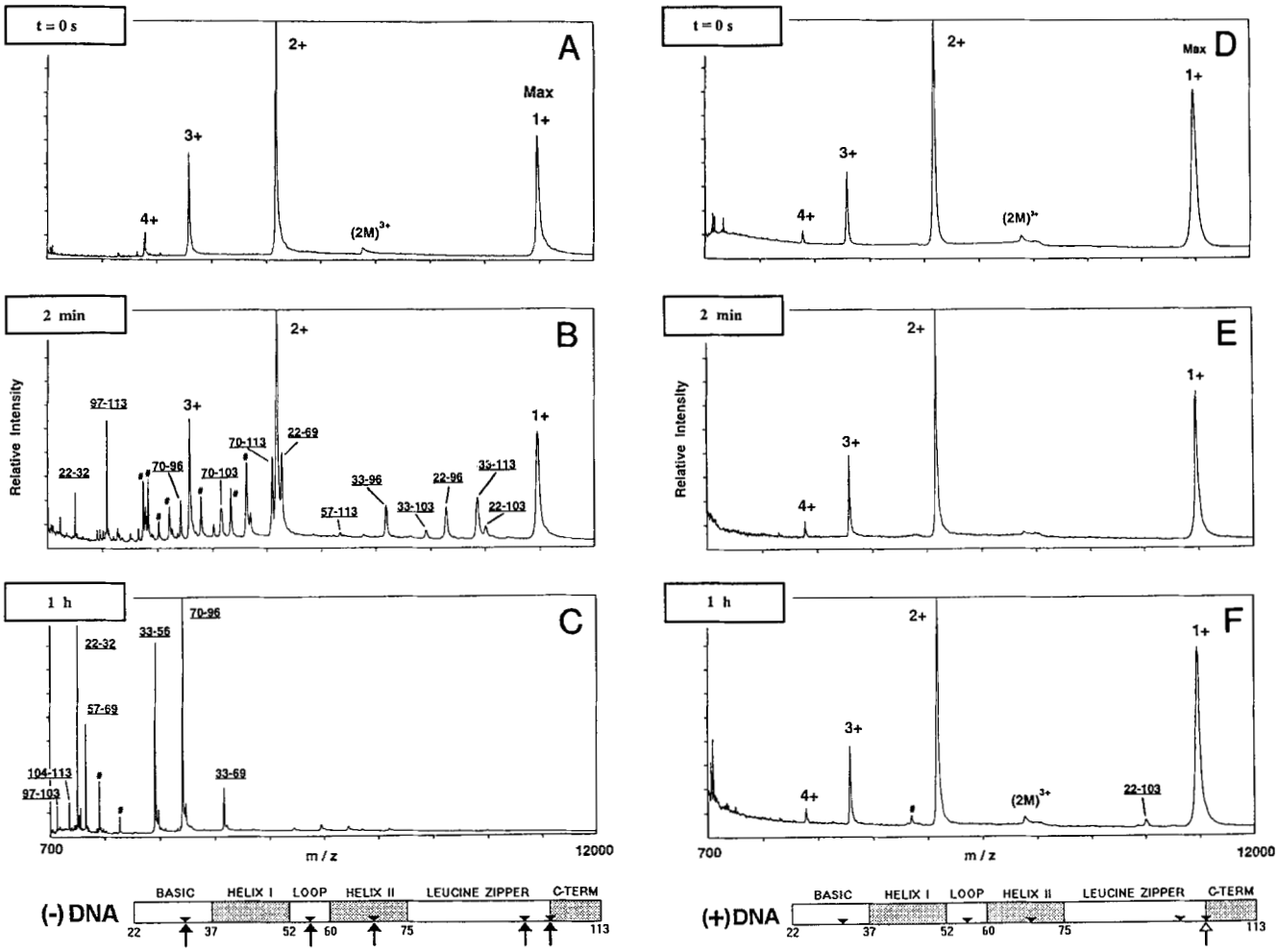

Fig. 3. V8 digestion of Max, (−) and (+) DNA. MALDI mass spectra of the products of a time-course V8 proteolysis of b/HLH/Z Max in the (**A, B, C**) absence and (**D, E, F**) presence of Max-specific DNA. Digests were performed in 15 mM KCl at pH 6 buffer and 25 °C (see Materials and methods). Three 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

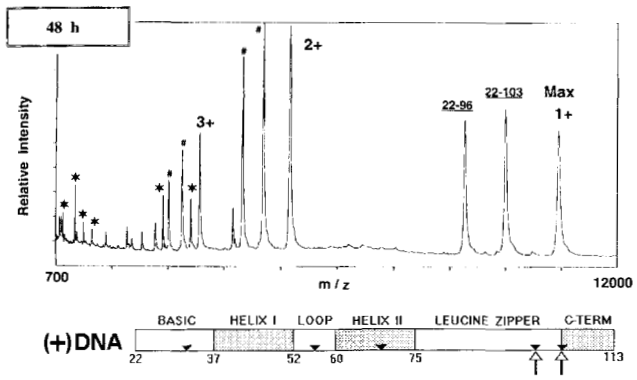

Fig. 4. V8 digestion of Max, (+) DNA at 48 h. MALDI mass spectrum of a 48-h V8 digest of the b/HLH/Z Max-DNA complex. Two peaks, 22-96 and 22-103, are the singly protonated V8 fragments of the protein. Peaks labeled with an asterisk represent complete V8 digest fragments. Linear diagram below the spectrum 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.

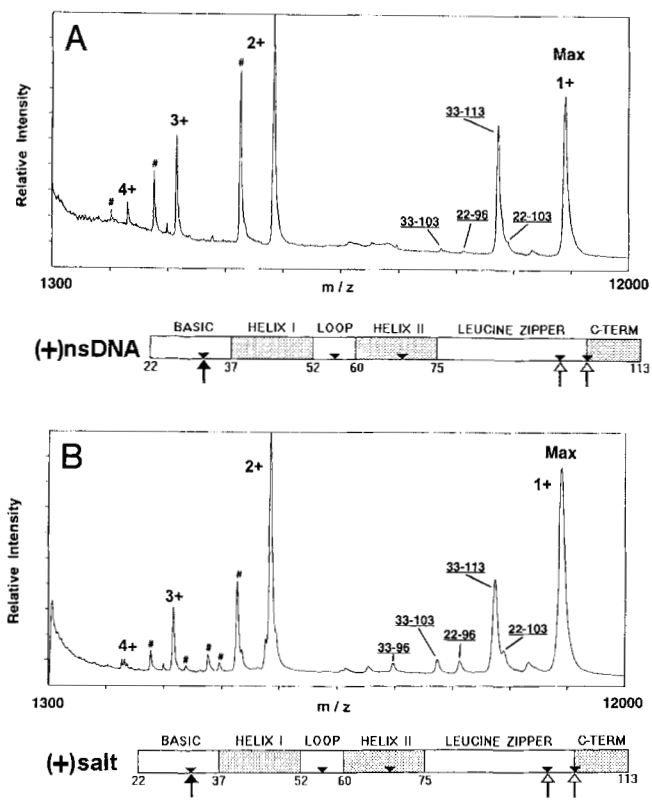

Fig. 5. V8 digestion of Max (+) nsDNA and Max at elevated ionic strength. **A:** MALDI mass spectra of a 5-min V8 digest of b/HLH/Z Max performed in the presence of nsDNA (TATA DNA; see Materials and methods). Digest was carried out at 15 mM KCl. Cleavage at the N-terminal of Max gives rise to the peptide 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.

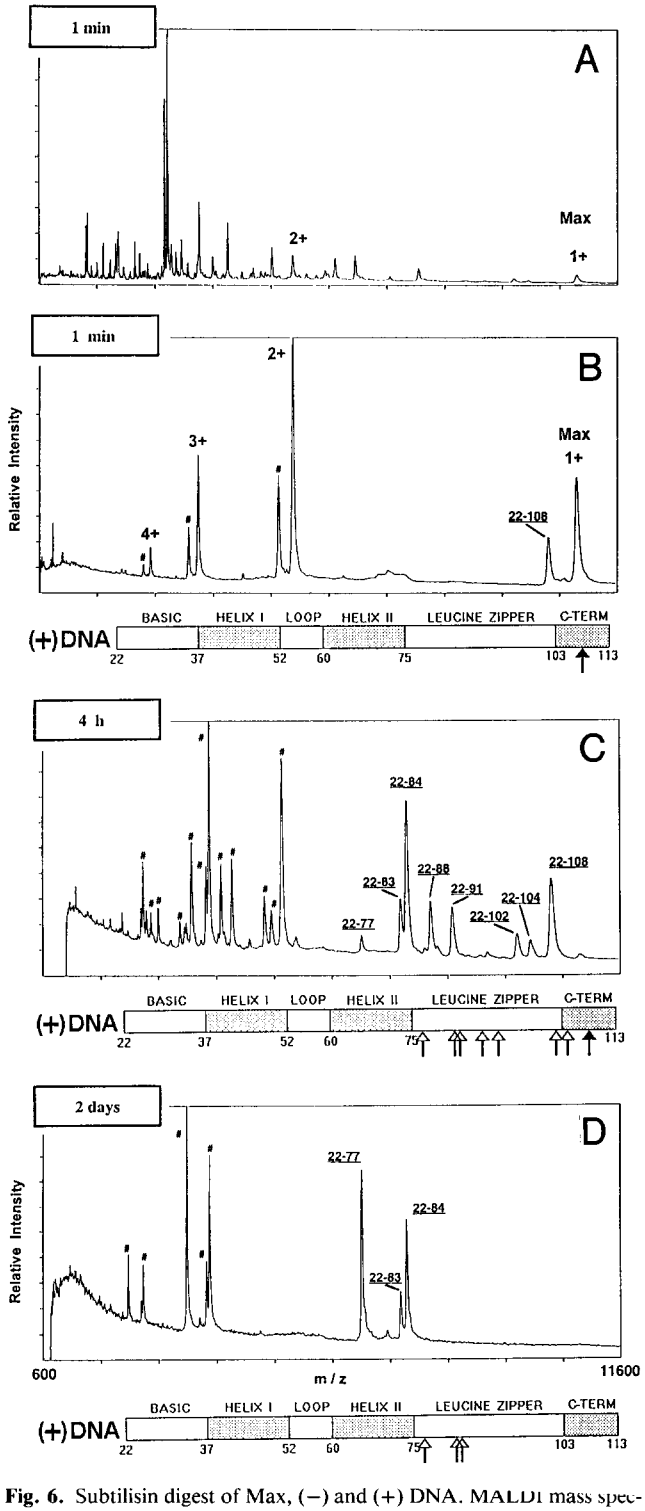

Fig. 6. Subtilisin digest of Max, (−) and (+) DNA. MALDI mass spectra of the subtilisin proteolysis of b/HLH/Z Max. **A:** The 1-min subtilisin digest in the absence of DNA. Extensive cleavage has occurred. The large number of fragment peaks are not labeled. **B, C, D:** The 1-min, 4-h, and 2-day subtilisin 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-

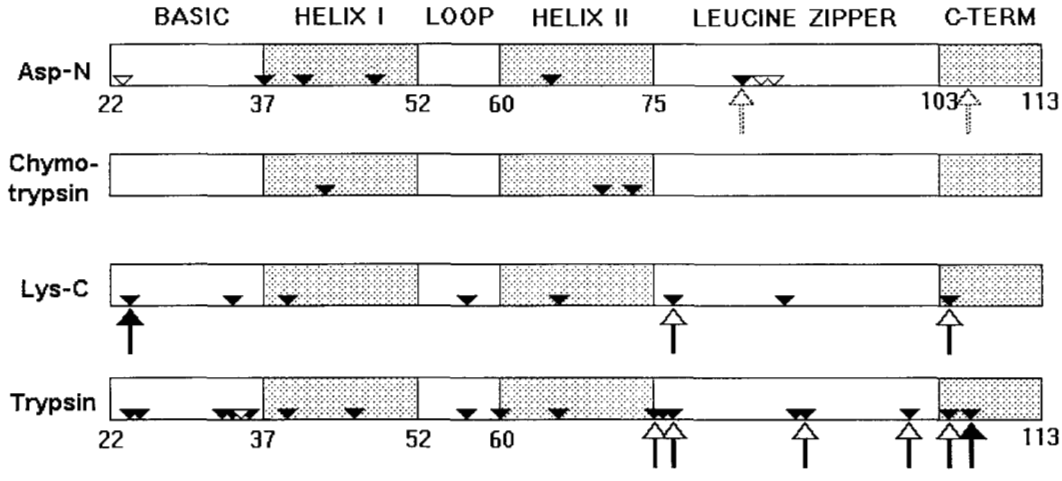

Fig. 7. Partial proteolysis of the b/HLH/Z Max–DNA complex. Results summarizing the proteolysis of b/HLH/Z Max in the presence of DNA by the proteases Asp-N, chymotrypsin, Lys-C, and trypsin. Small arrowheads inside the diagrams point to the respective potential proteolytic cleavage sites. Cleavage sites 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

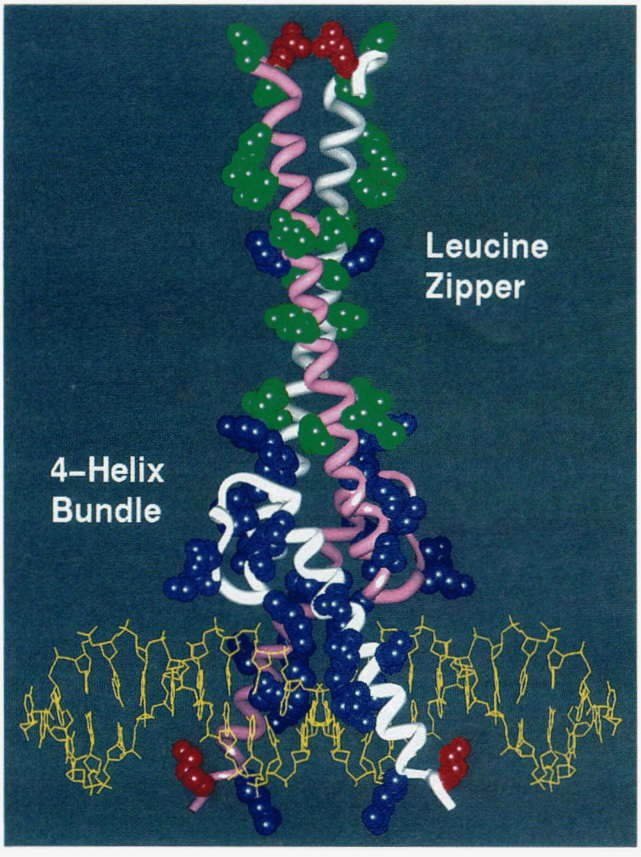

Fig. 8. Proteolytic protection map of the b/HLH/Z Max–DNA complex. The proteolytic protection map is a depiction of the combined results of analyses by MALDI-MS of a large number of proteolytic digestions performed on Max (in the presence of Max-specific DNA) with the endoproteases V8, Lys-C, Asp-N, chymotrypsin, trypsin, and subtilisin (see Results and Materials and methods). Using a color-coded measure of protection against proteolysis, the figure was created by superimposing the MALDI-MS proteolysis results onto a ribbon diagram of the X-ray crystal structure of the Max–DNA complex (Ferré-D'Amaré et al., 1993). The peptide backbone tracing the two Max monomers are outlined as white and pink ribbons. N-terminals (residue 22) are at the bottom; C-terminals (residue 107) are at the top. Coordinates of the last six residues (108–113) were not available because the X-ray crystallographic electron density map indicated that these residues were random coil. Amino acid residues that are proteolytic targets have their side chains highlighted as colored spheres in a space-filling representation. (All highlighted sites were shown to cleave rapidly in the absence of DNA.) The three colors reflect a relative measure of protection against partial proteolysis at the highlighted residue. Blue represents side chains that dramatically resist proteolysis over a 2–4-day period. Green indicates less protection, where complete or partial proteolysis at these sites has occurred over this time. Red represents the lowest level of protection, where complete cleavage at these sites occurred in less than 1 day. Protection from proteolytic cleavage at the "blue" sites together with cleavage at either a "green" or "red" site results in a truncated form of the protein that remains bound to DNA.

because of crystal packing, the ϵ-NH_2 of Lys 24 was interacting with a *neighboring* DNA molecule. However, it could not be determined whether Lys 24 was making contact with its *bound* DNA in the absence of crystal packing. Our proteolysis results show that Lys 24 is rapidly cleaved by Endo Lys-C. The resulting shortened protein (i.e., fragment 25–113) remains highly resistant to further Lys-C cleavage, indicating that DNA binding is maintained. Thus, we have shown that Lys 24 is not required to *preserve* DNA binding. We did not, however, determine whether Lys 24 was needed to *establish* DNA binding.

The central region

The second level of proteolytic protection is the region of Max that correlates to the four-helix bundle. Our results are consistent with the notion that this region is a highly compact, globular domain. The three conserved aromatic residues of the bundle (Phe 43, Tyr 70, Tyr 74) were highly protected against chymotrypsin cleavage, reflecting the inaccessibility of these residues to proteolytic attack. All of the Lys, Arg, Glu, and Asp residues of Helix I and II were also highly protected against cleavage. The eight-residue loop of the bundle showed a remarkable resistance to cleavage at Glu 56 (by V8) and Lys 57 (by Endo Lys-C and trypsin), and especially toward cleavage by subtilisin, a highly active protease with broad specificity. These results suggest that despite its solvent accessibility, the loop maintains a rigid structure in solution, in agreement with the X-ray crystallographic finding that the peptide backbone of the loop is well defined in the electron density map of the Max–DNA complex (Ferré-D'Amaré et al., 1993).

The C-terminal region

The lowest level of proteolytic protection of the Max–DNA complex was associated with the C-terminal region. Three conclusions can be deduced from the observed rates and patterns of proteolytic cleavage of this region. First, the complex showed a high susceptibility to cleavage from residues 107 to 113 (by subtilisin and trypsin), demonstrating the presence of a stretch of flexible polypeptide at the C-terminal. This result is consistent with the X-ray crystallographic finding that the C-terminal six residues were not visible in the electron density map of the Max–DNA complex (Ferré-D'Amaré et al., 1993). Second, the observed proteolytic "unzipping" of the complex is characteristic of a leucine zipper motif. (A similar pattern of proteolysis was observed to occur in another b/HLH/Z protein, SREBP-1 [sterol regulatory element binding protein; data not shown].) The sequential and gradual cleavage of the zipper can be understood in terms of its structure. The zipper consists of a coiled-coil arrangement of two amphipathic helices, resulting in a rigid structure that is stabilized by both hydrophobic and polar interactions between the two helices (Landschulz et al., 1988; O'Shea et al., 1989). The most accessible part of the zipper is its C-terminus where the two helices can more readily unfold and yield to proteolysis. Based on previous studies of leucine zipper domains, a strong polar interaction is predicted to occur between Lys 89 of one helix and Asp 84 of the other helix (and vice versa) (Baxevanis & Vinson, 1993). Our results show a high proteolytic protection of Lys 89 (against Lys-C and trypsin) and Asp 84 (against Asp-N), findings that may reflect the predicted interhelical electrostatic interaction between these two side chains. The "stalling" of proteolysis at residue 77 (by subtilisin) or at residue 75 (by trypsin) demonstrates that the region N-terminal to the zipper (the four-helix bundle) greatly resists proteolysis. This is the first report of an "unzipping and stalling" pattern of proteolysis of a leucine zipper. A third conclusion regarding the C-terminal region concerns the role of the leucine zipper on stabilization of the Max–DNA interaction. Partial or complete removal of residues of the zipper by proteolysis does not eliminate

DNA binding. This result indicates that the four-helix bundle is a highly stable structure that is sufficient to maintain DNA binding without the leucine zipper and supports the notion that the leucine zipper may be more important in defining dimerization specificity than it is in stabilizing the Max-DNA complex (Reddy et al., 1992). Finally, a note about the effects of higher-order oligomerization of Max dimers: tetramerization of other b/HLH/Z proteins has been observed and is believed to occur through an interaction between the leucine zipper domains of b/HLH/Z dimers (Ferré-D'Amaré et al., 1994 and references cited therein). Our proteolysis results do not allow us to draw any conclusions concerning the tetramerization of Max.

Evaluating the proteolytic protection assay

Several factors that impact upon the proteolytic protection assay are discussed below. These include specific versus nonspecific protein-DNA interactions, dynamics of the Max-DNA complex, and the use of MALDI-MS in proteolytic peptide mapping.

Specific versus nonspecific protein-DNA interactions

Our results indicate that the interactions between Max and sDNA lead to a significant protection against proteolysis of the Max dimer. Interactions between protein and DNA are usually categorized as either specific or nonspecific (Record et al., 1991). In the case of Max, there are highly specific interactions between the DNA-binding region of the protein and the oligonucleotide bases lining the major groove of the E-box recognition sequence (Ferré-D'Amaré et al., 1993). The dramatic increase in proteolytic protection of Max upon binding to DNA is an indication that specific protein-DNA interactions lead to a rigidification of the dimeric structure of the protein. There can also be weaker, nonspecific interactions that occur between positively charged groups of Max and negatively charged phosphate groups of DNA. These nonspecific protein-DNA interactions could shield the protein against proteolysis, complicating our simple correlation of proteolytic protection with structure. Because nonspecific protein-DNA interactions are known to weaken with increasing cation concentrations (Record et al., 1991), several proteolysis experiments were performed at elevated ionic strengths. However, our results have shown that raising the ionic strengths also leads to a tightening of the Max dimerization, masking any reduction in shielding that arises from nonspecific protein-DNA interactions. A compelling reason to suggest that the shielding does not greatly influence the patterns of proteolytic protection derives from experiments involving the proteolysis of Max carried out in the presence of nsDNA. Despite the high potential for shielding to occur between nsDNA and the highly basic N-terminal, this region of Max is rapidly and preferentially cleaved by either V8 (refer to Fig. 5A), Lys-C, or trypsin. These findings suggest that nonspecific protein-DNA interactions do not inhibit proteolytic activity in the basic N-terminal region, and by inference most likely across the rest of the protein.

Dynamics of the Max-DNA complex

If Max-DNA were a static complex, proteolytic fragments could only arise from digestion of the intact complex and there would be no ambiguity as to the origin of the fragments. However, protein-DNA complexes are known to be in dynamic equilibrium with unbound protein and DNA, so that proteolysis can occur from both the bound and unbound protein—leading to ambiguities in the interpretation of the data. We minimized this difficulty by maintaining an initial 50% molar excess of unbound DNA over Max-DNA complex in all of our experiments. The excess of unbound DNA ensured that virtually all of the Max molecules are complexed to DNA (see Note). At the same time, we maintained a 13-fold molar excess of total DNA over protease. This ensured that upon dissociation of the complex, Max is considerably more likely to encounter and reassociate with unbound DNA than it is to encounter protease. The success of this approach was confirmed by the long apparent lifetime (days) of the Max-DNA complex observed in our experiments.

MALDI-MS is well suited for the proteolytic protection assay

We have shown that MALDI-MS has adequate speed and mass accuracy to allow us to follow time-course proteolysis "on the fly" and to precisely map hundreds of complicated peptide digest mixtures. A comparable task handled by methods such as SDS-PAGE, liquid chromatography, and peptide sequencing would be tedious and prohibitively slow. As an alternative to the MALDI-MS technique, proteolytic digestions can be analyzed by electrospray ionization mass spectrometry. For example, limited proteolysis combined with electrospray ionization mass spectrometry was successfully applied to the analysis of structural changes in various forms of calmodulins upon calcium binding (Brockerhoff et al., 1992). However, the electrospray technique requires that samples for peptide mapping be free of involatile buffers and salts, necessitating thorough desalting. In addition, successful analysis of complex mixtures by electrospray often requires chromatographic separation of the mixture. In contrast, MALDI-MS is highly tolerant of buffers; salts and other biochemical additives and complex mixtures can be directly analyzed from digestion solutions.

Finally, we include a note regarding the quantitative aspects of our mass spectral data. The proteolytic protection assay is based on correlating proteolytic fragments to protein structure. The identities of the proteolytic fragments were determined from their masses, and the progress of the proteolytic digestions was determined by a semiquantitative evaluation of the relative heights of the fragment ion peaks. Although accurate quantitation of peptides and proteins by MALDI-MS can be difficult, we decreased our dependence on absolute quantitation by relying on a comparative analysis of the relative rates that proteolytic fragments appear and disappear (as mass spectral ion peaks) during the course of a digestion.

Conclusions

A simple biochemical method that combines enzymatic proteolysis and MALDI mass spectrometry was developed to probe aspects of the solution structures of DNA-binding proteins. The method is based on the notion that structural information can be inferred from a determination of protection against enzymatic proteolysis (as governed by solvent accessibility and protein flexibility). The procedure was applied to a study of the transcription factor Max. 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 reduction in rates together with the patterns of cleavage are consistent with homodimerization of Max through a predominantly hydrophobic interface. In the presence of Max-specific DNA, the protein becomes dramatically protected against proteolysis, exhibiting up to 100-fold reduction in cleavage rates, compared to the rates observed in the absence of DNA. The fragmentation patterns of proteolysis correlate well with those expected of a stable dimer bound to DNA at its basic N-terminals, in good agreement with the Max–DNA structure previously determined by X-ray crystallography. Protection is greatest in the N-terminal and helix-loop-helix regions of the protein, corresponding to the DNA-binding region and the globular four-helix bundle domain, respectively. An intermediate level of protection is found within the leucine zipper domain. The leucine zipper is distinguished by undergoing a sequential proteolytic cleavage that starts from the C-terminal of the zipper and terminates at the protease-resistant four-helix bundle. Proteolysis also indicates a high affinity of Max for its target DNA, which remains high even when the leucine zipper is proteolytically removed. Proteolytic protection is least near the N- and C-termini, indicating that these are the most flexible and accessible regions of the protein. The present biochemical results confirm and extend the X-ray crystallographic findings of the Max–DNA complex.

In addition to the study of the helix-loop-helix protein Max and its complex with DNA, the present method appears well suited for many other structural biological applications. These include the precise characterization of the boundaries of rigid protein domains (important for NMR and X-ray crystallography), the determination of binding specificity of DNA binding proteins, and the characterization of conformational changes induced in proteins by covalent modifications such as phosphorylation.

Materials and methods

Materials

A truncated form of the human Max protein (Max 22–113) was obtained by overexpression in *Escherichia coli* and purified as described previously (Ferré-D'Amaré et al., 1993). The protein was stored as a 3.6-mg/mL stock solution in 10% glycerol, 100 mM KCl, 10 mM HEPES-KOH, pH 7.5, buffer and kept frozen at −65 or −20 °C until used. Two DNA oligonucleotides were prepared synthetically (Ferré-D'Amaré et al., 1993), chromatographically purified, annealed, and stored in water at −20 °C. One of the oligonucleotides was Max-sDNA, a double-stranded, palindromic 14-mer with the sequence 5′-AGGTCACGTGACCT-3′ (the recognition E-box sequence is underlined). The other oligonucleotide was a double-stranded 14-mer (nsDNA), with the sequence 5′-AGCTATAAAAGGGC-3′ (TATA DNA). Endoproteases Glu-C (*Staphylococcus aureus* V8, catalog no. V5191), Lys-C (*Pseudomonas aeruginosa*, catalog no. V5131), and modified trypsin (bovine pancreas, catalog no. V5111) were purchased as sequencing grade quality from Promega (Madison, Wisconsin). Endoprotease Asp-N (*Pseudomonas fragi*, catalog no. 1420-488) and chymotrypsin (bovine pancreas, catalog no. 1418 467) were obtained as sequencing grade quality from Boehringer Mannheim (Indianapolis, Indiana). Subtilisin (*Bacillus subtilis*, catalog no. 165-905) was from Boehringer. The MALDI matrix used was 4HCCA (Beavis et al., 1992) (catalog no. 14550-5) and was from Aldrich (Milwaukee, Wisconsin).

Proteolytic digestions

Digestions were performed in the following manner. The stock solution of Max was thawed to room temperature and diluted with a 50 mM ammonium phosphate pH 6.0 buffer that includes 1 mM $Mg(OAc)_2$. A pH of 5.5 was used in the X-ray crystallographic study of the Max–DNA complex (Ferré-D'Amaré et al., 1993). A pH of 6 was adequate for high enzymatic activity toward Max (without DNA) for all six proteases used in this study (see Results). This pH also ensured a strong Max–DNA interaction. The final digest solution consisted of 30 μL of 30 μM Max (monomer concentration). Smaller solution volumes as well as lower protein concentrations can be used when less protein is available. For each protease experiment at least three 30-μL preparations were made: (−) DNA, (+) sDNA, and (+) nsDNA. Duplex DNA was added to the solution in a 1.5-fold molar excess over Max dimer. The solutions were allowed to incubate at room temperature for 10 min prior to the initiation of digestion. Digestion was started by the addition of an aliquot of an aqueous solution of a specific protease. The ratio of protease:Max ranged from 1:20 to 1:40 (w/w). All digests were performed at 25 °C. The time course MALDI-MS peptide analyses were performed in the following manner: 0.5-μL aliquots of the digest solution were withdrawn and mixed with 15 μL of MALDI matrix solution (see below) at 1-, 2-, 5-, 15-min; 1-, 2-, 4-, 8-h; and 1-, 2-day intervals following the start of digestion. Acidity of the matrix solution (pH < 3) completely quenched any further digestion.

MALDI-MS matrix preparation

The MALDI matrix solution was prepared by making a saturated solution of 4HCCA (about 25 mM) with 1:3:2 (v/v) formic acid:water:isopropanol (FWI). The FWI preparation of 4HCCA gave an excellent MALDI-MS response for observing protein and polypeptides with molecular masses over 3 kDa. A saturated preparation of 4HCCA in a 2:1 (v/v) mixture of 0.1% TFA(aq.):acetonitrile was also used and gave a good response for proteins as well as for the smaller polypeptides. The optimal MALDI-MS response for low molecular weight peptides (below 3 kDa) was observed with the use of a saturated solution of 4HCCA, prepared with a 2:1 (v/v) water:acetonitrile mixture. Polypeptides and proteins with masses above 3 kDa gave poor responses with the water:acetonitrile matrix solution (S.L. Cohen & B.T. Chait, manuscript in prep.) Following the mixing of an aliquot of digest sample with the appropriate matrix solution, a 0.5 μL portion of the resulting digest/matrix mixture was spotted onto an aluminum 10-position sample probe tip and allowed to air dry. To reduce excess salt in the resulting sample deposits, 1 μL of cold 0.1% TFA solution was placed over the dried matrix crystals on each spot of the probe tip and allowed to stand for 15 s before being removed by vacuum suction.

MALDI-MS instrumentation and data collection

MALDI-MS was obtained on a linear time-of-flight instrument. A full description of the instrument can be found elsewhere

(Beavis & Chait, 1989, 1990a, 1990b). Briefly, a multiple-sample probe tip was inserted into the time-of-flight instrument ion source and allowed to reach high vacuum. Background pressure within the instrument, measured by an ion gauge located below the source, was 3×10^{-7} torr. An Nd:YAG laser (Lumonics Inc., Ontario, Canada) was set to deliver 355-nm-wavelength pulses (approximately 10-ns duration) onto the sample at a rate of 2.5 Hz. Each laser shot produced a full mass spectrum. The spectra presented in this paper represent averages of 100–200 laser shots. The ion acceleration energy was +30 kV and the flight tube length was 2 m. Ion detection and signal amplification was through a conversion plate detector-dynode multiplier assembly (Beavis & Chait, 1991). The amplified signal was visually monitored with a digital oscilloscope (model 7200A, LeCroy Corporation, Chestnut Ridge, New York) and digitized by a transient recorder (LeCroy, model TR8828D) and stored as time-of-flight data on a computer (Vax 4000 workstation, Digital Equipment Corporation, Woburn, Massachusetts). The time-of-flight data was mass converted by use of either an added protein or peptide calibrant or with the use of digest ion peaks of known masses. A computer program facilitated the proteolytic mapping assignments. The Insight II program (Biosym Technologies, San Diego, California), run on an Indigo 2 workstation (Silicon Graphics, Mountain View, California), was used to create Figure 8.

Calculation of percentage of Max dimer bound to sDNA

A calculation of the percentage of Max dimer bound to Max sDNA under solution equilibrium conditions is shown below. The association between Max dimer and sDNA is given by Equation 1. The corresponding dissociation constant (K_D) relating equilibrium concentrations is given by Equation 2.

$$(Max)_2 + sDNA\ (\text{duplex}) \rightleftharpoons (Max)_2 \cdot sDNA\ (\text{duplex}) \quad (1)$$

$$K_D = \frac{[(Max)_2][sDNA]}{[(Max)_2 \cdot sDNA]}. \quad (2)$$

An estimate of the percentage of Max dimer bound to DNA at equilibrium can be calculated using the experimental initial concentrations of Max dimer and DNA (15 μM and 22.5 μM, respectively) and assuming a K_D of approximately 1 nM (A.R. Ferré-D'Amaré & S.K. Burley, unpubl. results).

Initial concentrations

$(Max)_2 = 1.50 \times 10^{-5}$ M
$sDNA$ (duplex) $= 2.25 \times 10^{-5}$ M
$K_D \cong 1 \times 10^{-9}$ M

Equilibrium concentrations

$(Max)_2 = 1.50 \times 10^{-5}$ M $- B$
$sDNA$ (duplex) $= 2.25 \times 10^{-5}$ M $- B$
B = conc. of the Max-DNA complex

From Equation 2,

$$K_D \cong 10^{-9}\ \text{M} = \frac{(1.50 \times 10^{-5} - B)(2.25 \times 10^{-5} - B)}{B}.$$

Solving for B,

$$B = [(Max)_2]_{Bound} = 1.4998 \times 10^{-5}\ \text{M}$$

$$\%Bound = \frac{[(Max)_2]_{Bound}}{[(Max)_2]_{Initial}} = \frac{1.4998 \times 10^{-5}\ \text{M}}{1.500 \times 10^{-5}\ \text{M}}$$

$$\times\ 100\% = 99.987\%.$$

Thus, under equilibrium conditions, a very small percentage (0.013%) of Max dimer is not bound to DNA.

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