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Structural Stability of Disulfide Mutants of Basic Pancreatic Trypsin Inhibitor: A Molecular Dynamics Study

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ABSTRACT **The structure and folding of basic pancreatic trypsin inhibitor (BPTI) has been studied extensively by experimental means. We report a computer simulation study of the structural stability of various disulfide mutants of BPTI, involving eight 250-psec molecular dynamics simulations of the proteins in water, with and without a phosphate counterion. The presence of the latter alters the relative stability of the single disulfide species [5-55] and [30-51]. This conclusion can explain results of mutational studies and the conservation of residues in homologues of BPTI, and suggests a possible role of ions in stabilizing one intermediate over another in unfolding or folding processes.** © 1996 Wiley-Liss, Inc.

Key words: BPTI, folding, unfolding, disulfide, molecular dynamics

INTRODUCTION

Little is known about the folding or unfolding pathways of most proteins. Yet information on the details of these pathways at the atomic level can provide understanding of how a one-dimensional chain of amino acids folds into a structurally stable and unique tertiary fold. The folding pathway of one small protein, basic pancreatic trypsin inhibitor (BPTI), has been extensively characterized experimentally. The structure of the 58-residue native protein is stabilized by three disulfide bonds. The folding pathway of the mature form of this molecule has been studied by determining[1,2] the role and order of the formation of native and nonnative disulfides in facilitating folding[1–3] and remains controversial. In the cell BPTI is expressed as a 100-residue pre-pro-BPTI, which contains additional cysteine residues, and their effect on the folding of the protein is now also being analyzed.[4,5]

The native BPTI structure (Fig. 1) has three disulfide bonds [5–55, 14–38, 30–51],[6] which have been replaced individually and in pairs by site-directed mutagenesis. Apart from the native disulfide configuration and the fully reduced molecule, six disulfide configurations can be formed by various combinations of native disulfides. The three double-disulfide cases [14–38, 30–51],[7–9] [5–55, 14–38][10] (A. A. Kossiakoff, personal communication), and [5–55, 30–51][11–13] maintain the native structure as well as its biological function. The three single-disulfide species are [5–55], [14–38], and [30–51]. The single disulfide bond of [14–38] is on the surface of the protein and is not likely to stabilize a well-defined structure. In the unfolding and folding of native BPTI, [14–38] is the first disulfide to break and the last to form.[3] The mutant species with only the single disulfide bond of [30–51], where all the other cysteines have been mutated to serine, maintains nativelike structure in solution for residues 19–36 (the β sheet) and 42–56 (the α helix). However, the amino terminus, including the trypsin binding loop, and the loop between residues 37 and 41 are disordered.[14,15] The single-disulfide species [5–55] is found by NMR to be folded in a native conformation and can inhibit trypsin both when the other cysteines are mutated to serines[16,17] and when they are mutated to alanines.[18] Both mutations make the structure less thermally stable than wild-type, with melting temperatures of about 15°C (serine mutants) and 40°C (alanine mutants) compared to the wild-type melting temperature of 104°C. The fully reduced molecule shows no stable secondary or tertiary structure on an experimentally measurable time scale.

Since protein unfolding is a process that occurs on the time scale of seconds, and the longest molecular dynamics (MD) computer simulations of proteins are of the order of nanoseconds (10^{-9} seconds), previous computer simulations of protein unfolding have used extreme temperatures to speed the unfolding process[19–21] or introduced additional virtual driving forces.[22] Both these techniques have been applied to study the unfolding and stability of BPTI. Daggett and Levitt[19,20] used high temperatures of 423 K and 498 K to compare the unfolding of the oxidized and reduced forms of BPTI. Hao and colleagues[22] applied an artificial centrifugal driving force to pull BPTI [14–38, 5–55] apart to an extremely extended form. Both techniques, high-temperature and artificial

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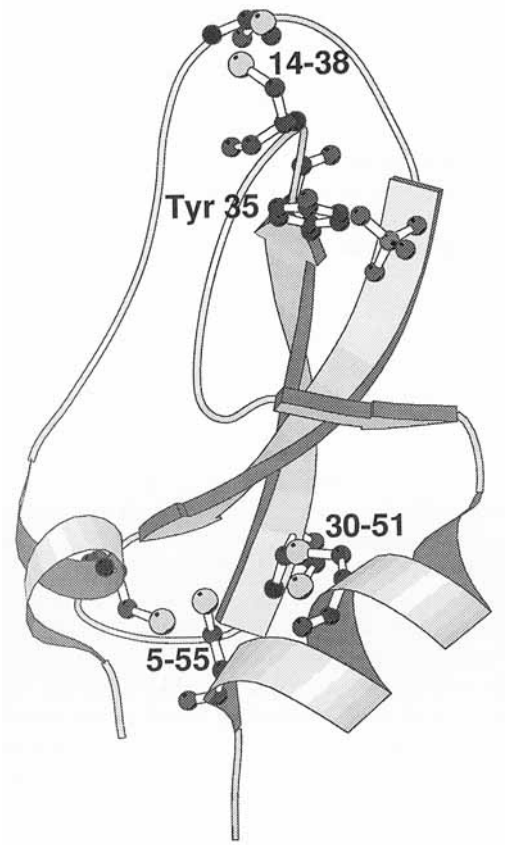

Fig. 1. A ribbon tracing[36] of the crystal structure of BPTI showing the location of the three disulfide bonds, Tyr 35 and the phosphate ion.

centrifugal force-driven unfolding, considerably accelerate the unfolding process, but at the cost of inducing a rather unphysical, nonrealistic unfolding pathway. An alternative theoretical treatment that avoids the time-scale problem at the expense of the neglect of solvent effects and nonharmonic dynamics is normal mode analysis. Tidor and Karplus[23] performed normal mode analysis for the three double-disulfide species to analyze the stabilization brought on the protein by the various disulfides.

In our study we propose yet another theoretical approach based on MD simulations and compare the structural integrity of two of the single-disulfide species, [5–55] and [30–51], that are structurally distinguishable experimentally with that of the native and a species without disulfides. By performing parallel MD simulations of BPTI mutants in aqueous solution at physically reasonable temperatures, all starting from the same native BPTI structure, we test whether MD can give an indication of the relative structural stability of these mutants. Experimentally, the [5–55] single-disulfide species stays in a native conformation, and the [30–51] single-disulfide species becomes more disordered. If the MD simulations yield the experimentally observed relative stability of the different mutants, analysis of the atomic trajectories may give an atomic picture of their differences in structural stability.

As a model system to see how far a molecule can drift in such a MD simulation over a given time period, we introduce a definitely unstable molecule: all the side chains are replaced with alanines except the four prolines and six glycines that are retained. We will refer to this molecule as BPTI-ALA.

METHODS

The starting point for all molecular dynamics simulations with the GROMOS[24] force field was an experimental NMR structure that had been equilibrated to 300 K in a bath of water, using periodic boundary conditions of a truncated octahedron, with a total of 2371 SPC/E[25] water molecules. When side chains were mutated into alanines, the atoms beyond the β carbons were just removed from the coordinate file. The initial velocities were taken from a maxwellian distribution at 300 K. Covalent bond lengths were constrained by using the SHAKE procedure[26] with relative tolerance of 10^{-4}. The time step used in the leapfrog integration scheme was 0.002 ps. A cutoff radius of 8.0 Å for the nonbonded interactions was used in conjunction with a pair list, which was updated every 10 time steps. The temperature of the system was maintained by weak coupling to a heat bath[27] at 300 K for 150 ps and then at 320 K for 100 ps, with a coupling constant of 0.1 ps, in each simulation. The temperature was increased slightly to facilitate unfolding, but not so much as to overwhelm the potential energy barriers that maintain the physical geometry of the molecule, and thereby distort the unfolding pathway. The backbone conformations of the resulting structures were then compared with the starting structure.

RESULTS AND DISCUSSION

Molecular dynamics simulations on each of the five molecules were performed. The structural stability of the three mutants is compared with that of the wild-type BPTI molecule as a lower limit of possible change and that of BPTI-ALA as an upper limit of possible change. A comparison of the starting and the final structures of each of the five simulations (Figure 2A–E) shows the difference in stability of the five molecules. The order from most to least stable is as follows:

1. Native
2. [5–55], [30–51]
3. No disulfide
4. BPTI-ALA

These results are consistent with experimental work on disulfide mutants in BPTI. Native BPTI shows motion only in the amino and carboxyl termini. The work by van Mierlo and colleagues[14–17] and Staley

and Kim[18] suggests that the [30–51] single-disulfide species should be less stable than the [5–55] single-disulfide species. From our simulations, these two molecules, although different, cannot be distinguished as far as overall structural stability is concerned. The molecule without disulfides is, however, considerably less stable than either of the single-disulfide species. As expected, BPTI-ALA is the least stable of the five structures.

An interesting feature of all five simulations is that there is very little motion either within or between pieces of secondary structure, even in the BPTI-ALA case. This is consistent with the theory that secondary structure forms a stable core of a protein, while the loops are more flexible, being the last part of the molecule to fold or the first part to unfold.

The fact that the simulations were unable to distinguish between the structural stability of the two single-disulfide species indicated either that the simulations were not of sufficient length to display a structural divergence of the two structures, or that something essential to the stability of these molecules was missing in the simulations. Reexamination of the crystal structure of BPTI, 5pti,[6] showed that a phosphate molecule that is closely coordinated by Tyr 35 and Arg 20 had not been included in the simulations. In 5pti, the deuterium atom of Tyr 35 DH is 2.0 Å away from one of the oxygens of the phosphate molecule, and two deuterium atoms at the end of the guanadinium group of Arg 20 are at 1.9 Å from two other oxygens of the phosphate molecule. Other amino acid residues within 5.0 Å of this phosphate are Ile 18, Gly 37, Ala 40, Asn 44, and Lys 46. This phosphate turns out to be present in all crystal forms of BPTI and has recently been implicated in facilitating crystal packing.[28] In the trypsin-BPTI complex[29] there are several water molecules placed in the phosphate binding site. It is likely that these placed water molecules could even represent a phosphate ion at this site, since the electron density generated by the latter would be similar to that of water molecules. In solution, a free phosphate ion would be very difficult to detect by protein NMR techniques. The possible presence of a phosphate ion in the solution near Tyr 35 suggests that this ion may play an integral structural role in the stability of BPTI. This role would likely become more pronounced when studying the relative stability of mutants such as the two single-disulfide species. If not the phosphate in particular, then the presence of a negative charge near Tyr 35 may be necessary, since BPTI has an overall molecular charge of $+6e$.

In order to investigate this hypothesis, three further simulations were set up. A phosphate ion was added to the native BPTI and the two single-disulfide species, [5–55] and [30–51]. The ion was placed where it was seen to bind in the 5pti crystal structure and five water molecules were removed from each system. The water molecules that were eliminated had atoms within the van der Waals radii of the atoms of the phosphate molecule. With the removal of five water molecules the number of nonhydrogen atoms in the system remains unchanged. The three systems were each energy-minimized for 100 steps with conjugate gradients to equilibrate the position of the phosphate ion, and then an identical simulation protocol, as previously described, was performed.

The addition of the phosphate ion did in fact induce a difference in the relative stability of these three molecules. Figure 2F–H shows the distance difference plots for these three simulations. The most dramatic change is observed in the mobility of the first 18 residues of BPTI relative to the rest of the molecule. Before addition of the phosphate the average displacement of the first 18 residues with respect to the rest of the molecule was 7.36 Å for the [5–55] molecule and 5.69 Å for the [30–51] molecule. With addition of the phosphate the relative displacement was reversed, the movement of the first 18 residues changed to 5.65 Å for the [5–55] molecule and to 6.56 Å for the [30–51] molecule. This is exactly the region which is found to be disordered experimentally in the [30–51] solution structure. The other region, which is more disordered in [30–51] than in [5–55], is the loop between residues 37–42 and again corresponds directly to what is seen to be disordered experimentally. In the native molecule the addition of the phosphate also reduces the motion seen in the amino terminal region of the molecule in the absence of phosphate. If one examines the electrostatic potential surface of wild-type BPTI, the region around the phosphate binding site is the most highly positively charged part of the molecule (Fig. 3). Therefore, it is not surprising that a counteracting negative ion would play a structural role in determining the relative stability of BPTI mutants.

Although there is no direct experimental evidence that changing the ionic environment of BPTI may alter the relative structural stability of the two single-disulfide species [5–55] and [30–51], extensive mutational studies have shown that alteration of several of the side chains surrounding the phosphate alters the relative prevalence of some of the folding intermediates.[30,31] These experiments were not always done with phosphate, but there was always a counterion such as Tris present. Tyr 35 has been studied the most extensively. All replacements of Tyr 35 greatly reduce the rate of disulfide formation and increase the rate of disulfide reduction. There is a crystal structure of Y35G[32] that crystallizes in a different space group than native BPTI and has nothing occupying the space where the phosphate is bound in the native structure. The Phe side chain in Y35F occupies virtually the same space as in the native structure but does not have the hydrogen

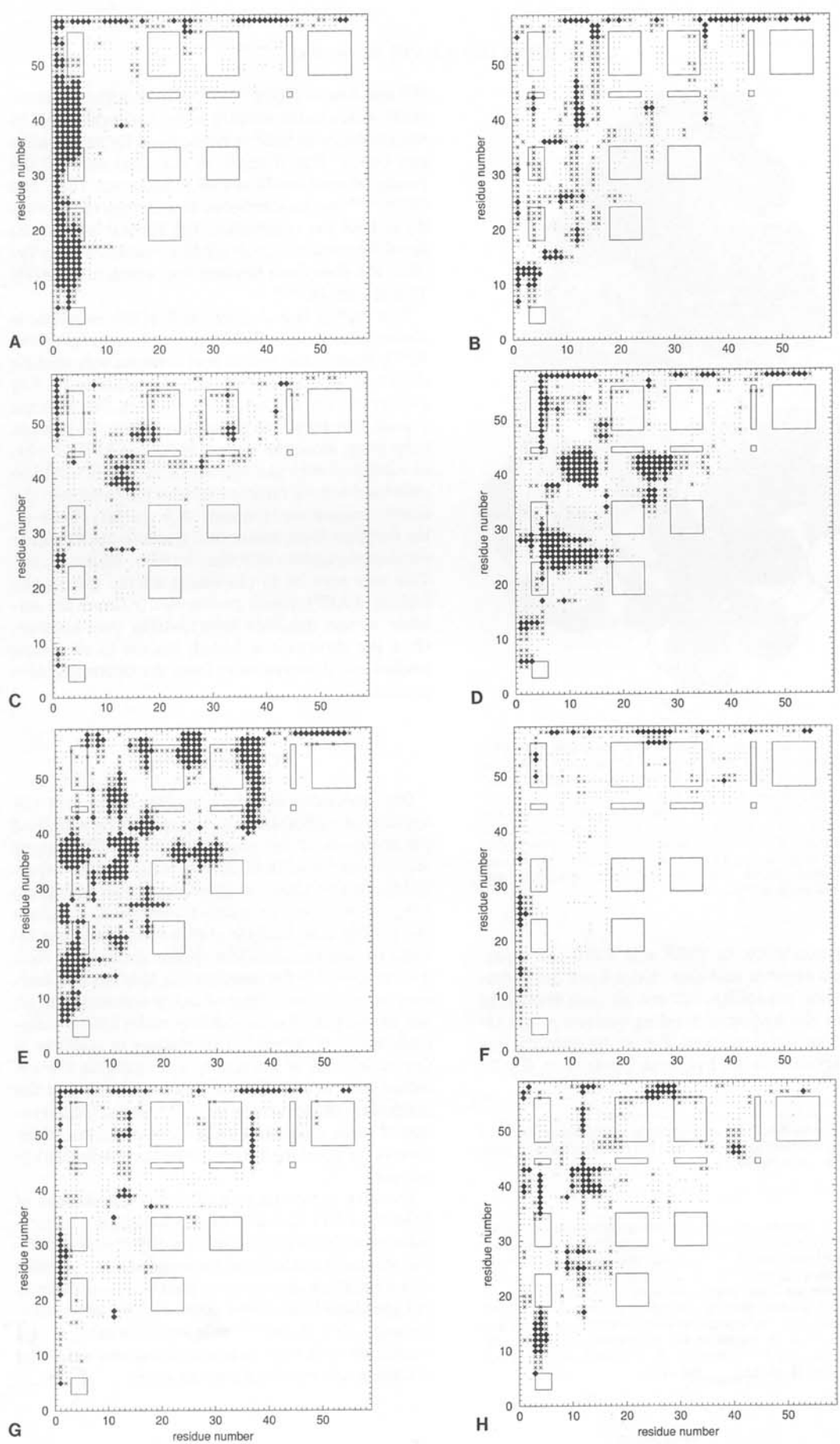

Fig. 2.

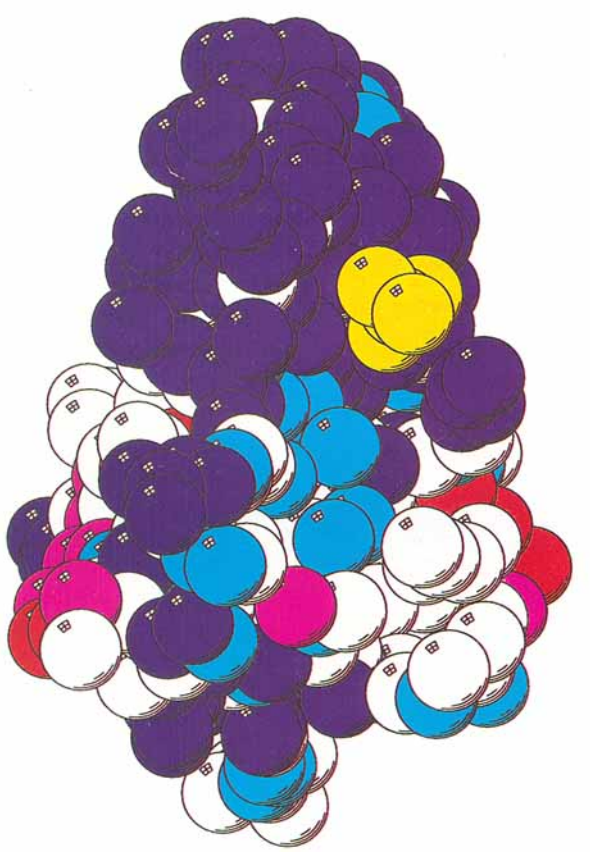

Fig. 3. The electrostatic potential was calculated from BPTI onto a Connolly surface within the MIDAS[37] program. The phosphate ion, which is shown in yellow, was not included in the calculation. This potential was then averaged onto each nonhydrogen atom. The coloring is by the average electrostatic potential at each atom from the corresponding area of the Connolly surface on a CPK surface.[36] Blue is an electrostatic potential of greater than 50*e*/Å, cyan is greater than 25*e*/Å, pink is less than −25*e*/Å, and red is less than −50*e*/Å. The orientation of the molecule is identical with that used in Figure 1.

bonding capability. In Y35N and Y35D the asparagine and aspartic acid side chains have the hydrogen bonding capability, but are not long enough to maintain the hydrogen bonding pattern of the tyrosine. The importance of Tyr 35 to stability has been attributed to its hydrogen bonds with Gly 37 NH and Asn 44 HD21[32–34]; however, although these residues are in the vicinity of the hydroxyl of Tyr 35 the geometry in 5pti[6] is not optimal for such hydrogen bonds. The changes in chemical shift of the amides of residues 37 and 40 on mutation Y35G and G37A[33,34] can be attributed to a change in the ability to bind the counterion. The folding is also hindered for mutants involving other residues than Tyr 35 in the phosphate binding site, which include Gly 37 and Asn 44.[33,34]

In a highly homologous (43% of the sequence is identical) protein to BPTI, another Kunitz inhibitor, APPI, most of the amino acid residues surrounding the phosphate binding site are identical (Ile 18, Arg 20, Tyr 35, Gly 37, Asn 44) to those in BPTI. In the crystal structure[35] no phosphate is seen. This is not surprising, since the overall charge of APPI is −3*e*, as compared with the +6*e* for BPTI, and it would be unfavorable for a further negative ion to exist in the closely packed environment of a crystal. However, the fact that these amino acid residues are so highly conserved suggests that they have an essential role. This role may be to coordinate an ion during the folding of APPI, which preferentially favors the stability of one disulfide intermediate over another. Once the structure is folded, the ion is no longer needed and it moves away from the electronegative protein.

CONCLUSIONS

Our molecular dynamics results imply that the addition of a phosphate ion preferentially stabilizes the structure of the single-disulfide BPTI mutant [5–55] over [30–51]. In light of these results, experimental observations could be interpreted as follows: the phosphate ion, or another counterion, stabilizes one folding intermediate over another and thus facilitates folding. Mutation of the amino acid residues responsible for coordinating this ion to a residue, which can no longer as easily accommodate an ion, causes the relative stability of the folding intermediates to be altered. This change in stability is due to the loss of the ability to coordinate the ion rather than to any internal structural role that the particular amino acid side chain plays. Conservation of these residues within other members of the Kunitz family of inhibitors further supports this hypothesis.

We have shown that parallel MD simulations of different BPTI mutants at physiological temperatures reproduces the experimentally observed relative structural stability of these molecules, provided that a negative counterion is included near its crystallographically observed position. The latter finding suggests a structural role for this weakly bound counterion with respect to stabilizing one unfolding or folding intermediate over another.

Fig. 2. Distance difference plot of the α-carbon backbone of the starting structure (s) of BPTI with the final structures (f) after 250 ps. The value plotted is

$$D_{ij} = | d_{ij}^{s} - d_{ij}^{f} |$$

where *i* and *j* indicate α carbons and the distance between atoms *i* and *j* in the starting structure and the final structure is indicated by d_{ij}^{s} and d_{ij}^{f}, respectively. In the plots, *filled diamonds* denote $D_{ij} \geq 3$ Å; *crosses* denote $D_{ij} \geq 2$ Å, and *dots* denote $D_{ij} \geq 1$ Å. The boxed regions are areas where one piece of secondary structure interacts with another piece of secondary structure or with itself. The figures show the difference between the starting structure and the various final structures of the trajectories. **A:** Native. **B:** [5–55]. **C:** [30–51]. **D:** No disulfide. **E:** BPTI-ALA. **F:** Native with phosphate (p). **G:** [5–55]p. **H:** [30–51]p.

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