The Potency and Specificity of the Interaction between the IA3 Inhibitor and Its Target Aspartic Proteinase from Saccharomyces cerevisiae*

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The yeast IA3 polypeptide consists of only 68 residues, and the free inhibitor has little intrinsic secondary structure. IA3 showed subnanomolar potency toward its target, proteinase A from Saccharomyces cerevisiae, and did not inhibit any of a large number of aspartic proteinases with similar sequences/structures from a wide variety of other species. Systematic truncation and mutagenesis of the IA3 polypeptide revealed that the inhibitory activity is located in the N-terminal half of the sequence. Crystal structures of different forms of IA3 complexed with proteinase A showed that residues in the N-terminal half of the IA3 sequence became ordered and formed an almost perfect α-helix in the active site of the enzyme. This potent, specific interaction was directed primarily by hydrophobic interactions made by the enzyme. This potent, specific interaction was directed primarily by hydrophobic interactions made by the enzyme. This was the first crystal structure to be determined for a gene-encoded aspartic proteinase inhibitor.

Aspartic proteinases participate in a variety of physiological processes, and the onset of pathological conditions such as hypertension, gastric ulcers, and neoplastic diseases may be related to changes in the levels of their activity. Members of this proteinase family, e.g. renin, pepsin, cathepsin D, and human immunodeficiency virus-proteinase are generally type-C viruses on the basis of their susceptibility to inhibition by naturally occurring, small molecule inhibitors such as the acylated pentapeptides, isovaleryl- and acetyl-pepstatin. However, the two most recently identified human aspartic proteinases, β-site Alzheimer’s precursor protein cleavage enzyme and β-site Alzheime’s precursor protein cleavage enzyme 2 (1, 2), are not inhibited by this classical type of inhibitor of this family of enzymes. Pepstatins are metabolic products produced by various species of actinomycetes and, as such, are not themselves gene-encoded. Protein inhibitors of aspartic proteinases are relatively uncommon and are found in only a few specialized locations (3). Examples include renin-binding protein in mammalian kidneys which intriguingly has now itself been identified to be the enzyme, N-acetyl-D-glucosamine-2-epimerase (4); a 17-kDa inhibitor of pepsin and cathepsin E from the parasite, Ascaris lumbricoides (5); proteins from plants such as potato, tomato, and squash (6, 7), and a pluriptotent inhibitor from sea anemone of cysteine proteinases as well as cathepsin D (8).

The IA3 polypeptide in yeast is an 8-kDa inhibitor of the vacular aspartic proteinase (proteinase A or saccharopepsin) that was initially described by Holzer and co-workers (9). The complete sequence of this 68-residue inhibitor has been elucidated (10, 11) and the inhibitory activity of IA3 has been shown to reside within the N-terminal half of the molecule (10, 12). We have recently solved the structure of the IA3-proteinase A complex (12), demonstrating that whereas free IA3 has little intrinsic secondary structure, residues 2–32 of the inhibitor, upon contact with proteinase A, become ordered and adopt a near perfect α-helical conformation occupying the active site cleft of the enzyme. This was the first crystal structure to be determined for a gene-encoded aspartic proteinase inhibitor complexed with its target enzyme. It was thus considered important to investigate further the role of the proteinase as a folding template and to attempt to establish the molecular features that enable this unprecedented mode of inhibitor-proteinase interaction to occur.

EXPERIMENTAL PROCEDURES

Protein Production and Purification—Proteinase A and other aspartic proteinases were obtained, and peptides were synthesized by solid-phase methods, as described previously (12). Genomic DNA was extracted from S. cerevisiae and the gene encoding IA3 was amplified specifically by polymerase chain reaction (PCR) using 5′-GCATAG-
ATACAGCAACAAAAAAGTG-3’ and 5’-CTCAGGACTCTCCTTCTTA- TGCCCGGC-3’ as forward and reverse primers. Mutations were introduced into the wild type sequence to generate clones encoding the chimera, (Gly)6 and K7M proteins (Table 1) by using the respective forward primers, 5’-AGGGTCACATGCTGAGTTCGTTTTTG-3’ and reverse, 5’-CTACGTATCAATTAACAGCCCCTGCAACATTTCCTTTGAGCTCTGAAA-3’ and reverse 5’-ACTGCGATCATCATCATAAAGCTGTCACTCATCCCTCT- CTTT; Mix: forward, 5’-AAGGCAATATGGACAGGACACG-3’ and reverse 5’-GAGAGATATACATATGGGAGGACACG-3’; D22L: forward, 5’-AAAGACAAGGACGG-3’ and reverse, 5’-TTTATGATGATGGCCAGTCAAGACAAGG-3’. PCR mutagenesis (13) using the mutagenic primer sets: K24M, forward, 5’-TGAAAATTTATCGGCCTTCACTAC-9GCGATGCAATGGTAGTGAGTGACGCTTTT-3’ and reverse, 5’-TACCTTGGCCAGCCCCTGCAACATTTCCTTTGAGCTCTGAAA-3’ and reverse, 5’-ACTGCGATCATCATCATAAAGCTGTCACTCATCCCTCT- CTTT; Mix: forward, 5’-AAGGCAATATGGACAGGACACG-3’ and reverse 5’-GAGAGATATACATATGGGAGGACACG-3’; and K7M, 5’-ATGGGAGGAGGCGGCGGCGGTGGAGGAGGCATATTTCAGAGCTCA-3’ and reverse, 5’-TACCTTGGCCAGCCCCTGCAACATTTCCTTTGAGCTCTGAAA-3’ and reverse, 5’-ACTGCGATCATCATCATAAAGCTGTCACTCATCCCTCT- CTTT; Mix: forward, 5’-AAGGCAATATGGACAGGACACG-3’ and reverse 5’-GAGAGATATACATATGGGAGGACACG-3’. The constructs encoding the K24M, K31M/K32M, Mix, D22L, and K7M mutants were each generated in two steps by overlapping PCR using the mutagenic primer sets. K24M, forward, 5’-GGGCGATCAGTGATGAGTGACGCTTTT-3’ and reverse, 5’-ACTGCGATCATCATCATAAAGCTGTCACTCATCCCTCT- CTTT; Mix: forward, 5’-AAGGCAATATGGACAGGACACG-3’ and reverse 5’-GAGAGATATACATATGGGAGGACACG-3’. Yeast IA3 Inhibitor

The wild type and mutant forms of IA3 were subcloned into the NdeI/Xhol sites of pET-22b (Novagen, Cambridge, United Kingdom), thus introducing a C-terminal Leu-Glu-His6 tag. Escherichia coli strain BL21DE3(pLysS) was transformed with wild type or mutant clones, grown by vapor diffusion under the conditions described previously for other IA3-protease A complexes (12). The initial solution was prepared at a molar ratio of inhibitor:protease of 5:1, and after separation from the excess of inhibitor by gel filtration on Sephadex G-50, was concentrated to 5 mg/ml by ultrafiltration. The mother liquor contained 0.14 mM isopropyl-1-thio-galactoside in 0.1 M MES buffer, pH 6.0. Data extending to 1.9 Å were collected at 100 K on beamline IXB at NSLS, Brookhaven National Laboratory, Upton, NY, using an ADSC 4K CCD detector. Data were processed with HKL2000 (16). The initial data set consisted of 217,446 reflections that could be scaled with Rmerge of 8.7% (last shell 34%) to yield 41,718 unique measurements. The co-]oun factors was 92.8% for the whole data and 75.5% for the final shell. The structure of protein A complexed with peptide 1 (12) was used as the initial model with replacement of Lys24 by Met. The structure was refined with CNS 1.0 (17) utilizing data extending to 2.0 Å resolution. The two first rounds of refinement included positional and B factor refinements and model adjustment, while solvent molecules were added in the third round. The final model contained the enzyme, residues 2–31 of the inhibitor and 243 water molecules. The final R factor was 19.84% and Ramrfree was 23.1%. The root mean square deviations for bond lengths and bond angles from ideality were 0.012 and 1.59 Å, respectively.

Modeling calculations were made on a Silicon Graphics Octane with a single R12000 processor using the Moloc modeling package. Individual amino acid side chains (K18M/D22L) in IA3 were changed with a command-line tool in Moloc. The whole IA3 structure was manually adjusted and a subsequent round of optimization, maintaining proteinase A and the remainder of IA3, fixed, resulted in low energy conformations for the newly introduced side chains. The new protein-inhibitor complex was checked for attractive and repulsive interactions, and allowed conformations, respectively.

RESULTS AND DISCUSSION

Interaction of Protein/Peptide Forms of IA3 with Target and Nontarget Enzymes—The nucleotide sequence encoding all 68 residues of IA3 was amplified by PCR and introduced into the pET-22b vector for expression in E. coli as described under “Experimental Procedures.” The recombinant protein that accumulated in E. coli was soluble and was purified to homogeneity from cell lysates by taking advantage of the His6 tag introduced from the pET-22b vector at the C terminus of the IA3 polypeptide chain (see “Experimental Procedures”). N-terminal analysis of one batch of the homogeneous wild-type inhibi- tor through 10 cycles of Edman degradation gave the sequence Asn-Thr-Asp-Glu-Gln-Lys-Val-Ser-Glu-Ile, which is exactly coincident with that predicted by the DNA sequence for residues 2–11 (Table 1) (11) and indicates that the initiator Met residue had been removed during the accumulation of this batch of recombinant protein in E. coli. Analysis of a separate batch of recombinant wild-type inhibitor by MALDI-TOF mass spectrometry gave a mass of 8772 Da, identical with that predicted (8772 Da) for the IA3 sequence plus the C-terminal extension of −Leu-Glu-His6 introduced from the pET-vector.

The KI value determined at pH 5.1 for the inhibition of yeast proteinase A by this C-terminal tagged, wild-type recombinant protein (wild-type in Table 1) was comparable to that reported previously at the same pH for the naturally occurring protein purified from S. cerevisiae (14). It is readily apparent then that the introduction of the extra −Leu-Glu-His6 residues at the C terminus of the recombinant inhibitor did not have any significant detrimental effect on inhibitory potency. Since the target proteinase is unlikely ever to encounter a pH as low as 3.1 in its cellular environment, attempts were also made to determine inhibition constants at higher pH values such as 4.7 and 6.0. In both cases, the interaction with proteinase A was so tight that the KI values lay at or beyond the limits of accurate determination using the available assay methodology and so were estimated to be <0.1 nM.

A synthetic peptide which spanned residues 2–34 of the IA3 sequence (Peptide 1 in Table II) was found to have inhibitory potency against yeast proteinase A at pH 3.1 and 4.7 compa-
Yeast IA$_3$ Inhibitor

Recombinant protein forms of IA$_3$ consisting of 68 residues plus a ~Leu-Glu-(His)$_8$ tag at their C terminus were produced in E. coli and purified to homogeneity. The sequence of residues 2–32 (only) of each protein is given.

### Table I
Reactions between yeast proteinase A and protein forms of IA$_3$

| Identifier       | Sequence | $K_i$ (nM) | $K_i$ (nM) |
|------------------|----------|------------|------------|
| Wild-type        | NTD QGKV SKEKL QGDAA VVSDDF FKKZA | 1.1 ± 0.4  | <0.1       |
| Chimeric         | NTD QGKV SPIKL QGDAA VVSDDF FKKZA | 8 ± 1      | <0.1       |
| (Gly)$_9$        | NTD QGKV SIPLK QGDAA VVSDDF FKKZA | 1.3 ± 0.4  | <0.1       |
| K7M              | NTD QGKV SIEKFQ SKEKL VVSDDF FKKZA | 10 ± 0     | 1.0 ± 0.7  |
| K24M             | NTD QGKV SIEKFQ SKEKL VVSDDF FKKZA | 2 ± 1      | <0.1       |
| K31M/K32M        | NTD QGKV SIEKFQ SKEKL VVSDDF FKKZA | 0.9 ± 0.3  | <0.1       |
| MIX              | NTD QGKV SIEKFQ SKEKL VVSDDF FKKZA | 8 ± 2      | 0.6 ± 0.3  |
| D22L             | NTD QGKV SIEKFQ SKEKL VVSDDF FKKZA | 8 ± 2      | 0.6 ± 0.3  |
| K18M/D22L        | NTD QGKV SIEKFQ SKEKL VVSDDF FKKZA | 8 ± 2      | 0.6 ± 0.3  |

$^a$I$_{50}$ value.

### Table II
Reactions between yeast proteinase A and synthetic peptide forms of IA$_3$

| Identity No. | Sequence | $K_i$ (nM) | $K_i$ (nM) |
|--------------|----------|------------|------------|
| 1            | NTD QGVN SKEKL QGDAA VVSDDF FKKZA | 2 ± 0.6  | <0.1       |
| 2            | NTD QGVN SKEKL QGDAA VVSDDF FKKZA | 3 ± 1    | ND$^b$     |
| 3            | NTD QGVN SKEKL QGDAA VVSDDF FKKZA | 4 ± 1    | 0.8 ± 0.2  |
| 4            | NTD QGVN SKEKL QGDAA VVSDDF FKKZA | 8 ± 2    | 6 ± 1.5    |
| 5            | NTD QGVN SKEKL QGDAA VVSDDF FKKZA | 85 ± 20  | 29 ± 8     |
| 6            | NTD QGVN SKEKL QGDAA VVSDDF FKKZA | 50 ± 50  | 500 ± 120  |
| 7            | NTD QGVN SKEKL QGDAA VVSDDF FKKZA | 15 ± 4   | 2 ± 0.4    |
| 8            | NTD QGVN SKEKL QGDAA VVSDDF FKKZA | 5 ± 0.6  | 0.8 ± 0.3  |
| 9            | NTD QGVN SKEKL QGDAA VVSDDF FKKZA | 25 ± 8   | 5 ± 1      |
| 10           | NTD QGVN SKEKL QGDAA VVSDDF FKKZA | 700 ± 200| 400 ± 50   |
| 11           | NTD QGVN SKEKL QGDAA VVSDDF FKKZA | 120 ± 0  | 60 ± 9     |
| 12           | NTD QGVN SKEKL QGDAA VVSDDF FKKZA | 240 ± 50 | 60 ± 9     |
| 13           | NTD QGVN SKEKL QGDAA VVSDDF FKKZA | 1,000 ± 300 | 1,000 ± 300 |
| 14           | NTD QGVN SKEKL QGDAA VVSDDF FKKZA | No inhibition | No inhibition |

$^a$ND, not determined.
$^b$NI, no inhibition at a final concentration of 2 μM.

### Table III
Aspartic proteinases unaffected by the IA$_3$ inhibitor

| Enzyme                     | Assay pH | Enzyme | Assay pH |
|----------------------------|----------|--------|----------|
| Yapsin                     | 5.3      | Human pepsin | 4.7 |
| Penicillopepsin            | 4.7      | Human gastricin | 4.7 |
| Endothiapepsin             | 4.7      | Human cathepsin D | 4.7 |
| Mucor miehei proteinase    | 4.7      | Human cathepsin E | 4.7 |
| Mucor pusillus proteinase  | 4.7      | Human napsin | 4.7 |
| S. lignisodium proteinase A| 4.7      | Calf chymosin | 4.7 |
| Glomerella cingulata proteinase | 4.7 | Plasmepsin II | 4.4 |
| Cyspepsin                  | 5.0      |        |          |

The entire sequence of each peptide is given in full. Z = L-norleucine.

Table is rable to those of the naturally occurring and wild-type recombinant protein forms of IA$_3$, described previously (12, 14). In contrast, peptide 1, at a concentration of 2 μM, had no significant ability to inhibit any one of a number of other aspartic proteinases from a wide range of other species (Table III). These have considerable sequence and structural similarities to yeast proteinase A and included yapsin 1 (a membrane-attached aspartic proteinase also from S. cerevisiae (15) and other enzymes of fungal, mammalian, parasite (plasmepsin II from Plasmodium falciparum (18) and plant (cyprosin from Cynara cardunculus (19)) origin. Thus, IA$_3$ is a potent specific inhibitor directed solely against its target enzyme, yeast proteinase A. Since peptide 1 had no effect on the non-target enzymes listed in Table III, reciprocally, the effect of a number of other enzymes listed in Table III, because these enzymes cut the polypeptide effectively as a substrate. Consequently, the −Ala$_{29}$Phe$_{30}$− and −Glu$_{10}$Ile$_{11}$− sites that were cleaved by the non-target proteinases were changed singly and in concert to introduce residues at the P$_i$ position which were known from extensive previous studies (e.g. Refs. 20 and 21) to be refractory to cleavage by such enzymes as human pepsin and cathepsin D. The resultant peptides (2 and 3, Table II) were just as potent as peptide 1 as inhibitors of proteinase A but still showed no significant ability to inhibit, e.g. human pepsin or cathepsin D at concentrations as high as 5 μM. Although cleavage of peptides 2 and 3 between residues −Val$_{29}$Phe$_{30}$− and...
sequence, and indicating once again that the initiator Met1 residue had been removed by E. coli proteinases. Consistent with this, analysis by MALDI-TOF mass spectrometry gave a mass of 8502 Da, identical to that (8502 Da) predicted for the sequence of residues 2–68 plus the −Leu-Glu-His5 tag of the chimeric protein. Remarkably, this chimeric protein with 9/34 (26%) of its residues exchanged was still almost as effective as an inhibitor as the wild-type protein, with its interaction at pH 4.7 still being so tight as to lie at or beyond the limits of accurate determination (Table I). This result, together with the deletion experiments described above with the peptide form of IA3, suggests that, for effective inhibition, backbone atoms contributed by residues 2–10 are essential but that the (side chain) identity of the individual amino acids in these positions is of lesser importance. On this basis, we replaced residues 2–10 of the natural sequence with nine glycine residues ([Gly]9 mutant, Table I) and purified the resultant protein to homogeneity.

To investigate the importance of the individual side chains of these residues, alterations in sequence were introduced into the full-length protein form of IA3 at its N terminus by PCR mutagenesis as described under "Experimental Procedures." Initially, residues 2–10 of the wild-type IA3 sequence were replaced with Gly-Gly-His-Asp-Val-Pro-Leu-Thr-Asn-Ile, identical to that predicted by the DNA sequence, and indicating once again that the initiator Met1 residue had been removed by E. coli proteinases. Consistent with this, analysis by MALDI-TOF mass spectrometry gave a mass of 8502 Da, identical to that (8502 Da) predicted for the sequence of residues 2–68 plus the −Leu-Glu-His5 tag of the chimeric protein. Remarkably, this chimeric protein with 9/34 (26%) of its residues exchanged was still almost as effective as an inhibitor as the wild-type protein, with its interaction at pH 4.7 still being so tight as to lie at or beyond the limits of accurate determination (Table I). This result, together with the deletion experiments described above with the peptide form of IA3, suggests that, for effective inhibition, backbone atoms contributed by residues 2–10 are essential but that the (side chain) identity of the individual amino acids in these positions is of lesser importance. On this basis, we replaced residues 2–10 of the natural sequence with nine glycine residues ([Gly]9 mutant, Table I) and purified the resultant protein to homogeneity. No attempts were made to sequence this protein because of the plethora of glycine residues but MALDI-TOF mass spectrometry gave a mass of 8124 Da, identical to that (8124 Da) predicted by the nucleotide sequence but, once again, lacking the initiator Met1 residue. The yield of this mutant protein obtained from E. coli was about 5-fold lower than that obtained for the wild-type (and other mutant) sequence(s). This drastic introduction of nine consecutive glycine residues resulted in a poorer inhibitor with a Kd of 40 nM at pH 3.1 (Table I). However, at pH 4.7, the (Gly)9 mutant protein was still a very effective inhibitor, with its potency quantified at around 1 nM (Table I). Thus, the main chain atoms at the N terminus of the 2–34 sequence would appear to be the major contributors to inhibitory potency from this region with only minor influences being introduced by the individual residue side chains. This was substantiated further by replacement of individual residues, for example, the Lys7 residue was replaced with methionine which is quasi-isosteric with lysine but lacks the e-NH2 group. This K7M mutant inhibitor was just as potent as the wild-type protein (Table I).

Truncation of the inhibitory sequence of residues 2–34 at its C terminus also resulted in a progressive loss of inhibitory potency (compare peptide 1 and peptides 9, 10, 11, 12, and 13, Table II). Replacement of Lys31 by Met (K24M mutant) and Lys31 + Lys32 (together) in the double mutant K31M/K32M again had no significant effect on the inhibitory potency of the resultant inhibitors toward proteinase A (Table I). The structure of the K24M mutant complexed with proteinase A was solved at 2 Å resolution and refined to an R factor of 19.84% (see "Experimental Procedures"). Comparison with the structures reported previously (12) for the K31M/K32M protein form and for the peptide 1 form of IA3 complexed with proteinase A (Protein Data Bank accession codes 1dp5 and 1dqj) revealed that, in all three cases, residues 2–32 of the inhibitor had adopted a near-perfect α-helical conformation in the active
Lys32. This was achieved by the inclusion of Nle33 (a synthetic isostere of the natural Met residue at this location) and Ala34 in protein and peptide form(s) of the inhibitor. The IA3 helix is Lys32 in the crystal structure of the K24M mutant complex or C-terminal end to stabilize the inhibitory helix. No electron density within the inhibitory helix, thereby stabilizing the helix be for the backbone atoms to satisfy the H-bonding arrange-

sion of the negative charge from the macromolecular dipole.

The structures all reveal that the main chain carbonyl and amido moieties of residues 2–10 of the IA3 polypeptide are involved in H-bond formation with one another within the helix of the inhibitor but the side chains of these residues make no significant contacts with the protease, with the exception of Val10 which is involved in hydrophobic interactions (described later). In the sequence of the chimera inhibitor described earlier (Table I), Val10 was replaced with Leu which may be able to make hydrophobic contacts with the enzyme requiring only minor re-adjustment so that the derived Kᵢ value was not significantly perturbed. The binding energy of these contacts is clearly lost in the (Gly)9 mutant protein but since the side chains of all of the other residues in the 2–10 sequence point largely into solvent, the still considerable inhibitory potency of the “half-sized” peptides was able to inhibit protease A when added singly or in combination with each other at a variety of molar ratios. Thus a contiguous sequence is necessary for IA3 to inhibit protease A by forming the amphipathic helix (Fig. 1). The residues located on the hydrophobic face make extensive hydrophobic contacts with cognate residues in protease A. Particularly noticeable in this regard (Fig. 1) is the “cluster” arrangement of Val10-X-X-hydrophobic-Phe12 toward the front end of the helix and Val26-X-X-hydrophobic-Phe10 at the back end of the inhibitory sequence. In the front end cluster, replacement of Val10 or Ile11 individually by Ala had minor effects on inhibitory potency (compare peptides 15 and 16 with peptide 9, Table IV). However, deletion of the benzene ring of phenylalanine at position 12 resulted in a considerably larger drop in potency in the resultant Ala-containing peptide (peptide 17, Table IV), emphasizing the importance of van der Waals interactions of this benzene ring with its hydrophobic environment (Fig. 2). The peptide carrying the double replacement of Val10 with F12A (peptide 18, Table IV) had lost much of its inhibitory potency and the double mutant peptide containing −Ala11−Ala12− (peptide 19) was virtually ineffective as an inhibitor, even at pH 4.7. The triple mutant in which all three of the front-end cluster residues were changed to −Ala−X-X-Ala11−Ala12− (peptide 20, Table IV) was completely inactive as an inhibitor.

A similar response was quantified when the residues contributing to the −Val26−X-X-hydrophobic-Phe30− cluster at the back end of the helix were replaced. Substitution of Phe30 by Ala, Gly, or Lys (compare peptides 21 and 22/23 with peptide 10, Table IV) resulted in a significant loss in potency, again emphasizing the contribution to binding by appropriate positioning of the large benzene ring of the side chain of Phe30. In contrast, replacement of Val26 by Ala did not diminish inhibitory potency. Rather, it appeared to improve the binding interaction at pH 3.1 marginally (peptide 24, Table IV). Replacement of the -CH₃ side chain of Ala with the -CH₂-COOH side chain of an Asp at position 26 diminished the inhibitory potency at pH 4.7 by about 70-fold (compare peptides 25 and 24, Table IV), commensurate with the introduction of a hydrophilic

| Identity No. | Sequence | pH 3.1 | pH 4.7 |
|-------------|----------|--------|--------|
| 9. NTD QQKVS EIFQS SKEKL QGDAR VVSDA FKK | 15 ± 4 | 2 ± 0.4 |
| 10. NTD QQKVS EIFQS SKEKL QGDAR VVSDA FKK | 70 ± 15 | 8 ± 2 |
| 11. NTD QQKVS EIFQS SKEKL QGDAR VVSDA FKK | 60 ± 25 | 7 ± 2 |
| 12. NTD QQKVS EIFQS SKEKL QGDAR VVSDA FKK | 400 ± 40 | 55 ± 11 |
| 13. NTD QQKVS EIFQS SKEKL QGDAR VVSDA FKK | 2,100 ± 300 | 330 ± 80 |
| 14. NTD QQKVS EIFQS SKEKL QGDAR VVSDA FKK | 800 ± 200 |
| 15. NTD QQKVS EIFQS SKEKL QGDAR VVSDA FKK | 25 ± 8 | 5 ± 1 |
| 16. NTD QQKVS EIFQS SKEKL QGDAR VVSDA FKK | 175 ± 60 | 35 ± 6 |
| 17. NTD QQKVS EIFQS SKEKL QGDAR VVSDA FKK | 200 ± 70 | 75 ± 14 |
| 18. NTD QQKVS EIFQS SKEKL QGDAR VVSDA FKK | 200 ± 90 | 76 ± 9 |
| 19. NTD QQKVS EIFQS SKEKL QGDAR VVSDA FKK | 10 ± 2 | 6 ± 1 |
| 20. NTD QQKVS EIFQS SKEKL QGDAR VVSDA FKK | 150 ± 55 | 400 ± 130 |
| 21. NTD QQKVS EIFQS SKEKL QGDAR VVSDA FKK | NI* | 700 ± 120 |

* NI, no inhibition at a final concentration of 2 μM.
side chain into a hydrophobic environment. However, the $K_i$ value measured at pH 3.1 for peptide 25 was tighter than that derived at pH 4.7 (Table IV). Of all the inhibitors listed in Tables I, II, and IV, this was the only occasion when such an effect was observed and most likely is a reflection of the Asp side chain in its protonated and therefore uncharged form being less unfavorable in its contact with the hydrophobic environment offered by the enzyme.

The double mutant peptide carrying the V26D/F30K substitutions was completely ineffective as an inhibitor (peptide 26, Table IV), indicating that introduction of two hydrophilic, charged residues was highly unfavorable since there are no H-bond partners available in the enzyme to compensate for desolvation of the two side chain functions. However, amphipathic helices are often stabilized by electrostatic interactions between residues at positions $i$ and $i + 4$ (23) and, indeed exactly such a salt bridge is present between the Lys 24 and Asp28 residues on the hydrophilic face of the IA3 helix when complexed with its target proteinase (Fig. 1). However, the attempt to encourage Asp26 and Lys30 to interact with one another to form an additional salt bridge in the V26D/F30K double mutant peptide was clearly not tolerated on the hydrophobic face of the amphipathic helix in the active site cleft of the enzyme. A further mutant was also constructed in which the sequence of $-$Ser$^{27}$-Asp$^{28}$-Ala$^{29}$-Phe$^{30}$-Lys$^{31}$-Lys$^{32}$ $-$ was shuffled to $-$Lys$^{37}$-Ala$^{38}$-Asp$^{39}$-Lys$^{40}$-Phe$^{41}$-Ser$^{42}$ $-$ in the protein form of the IA3 inhibitor (Mix in Table I). In this arrangement, Val26 was retained but the salt bridge between Lys24 and Asp28 on the hydrophilic face of the helix was disrupted and the crucial Phe30 residue was replaced by lysine, as in peptide 23. The resultant mutant protein (Mix, in Table I) was purified to homogeneity from E. coli and found to have a $K_i$ value at pH 4.7 comparable to that observed for the single F30K mutant peptide (peptide 23, Table IV). This might be interpreted to indicate that the salt bridge interaction between Lys24 and Asp28 on the hydrophilic surface of the IA3 helix is, not unexpectedly, weak.

Central Residues in the Inhibitor Helix—The “centerpiece” of the inhibitory 2–34 residues of IA3 is the $-$Lys$^{18}$-Leu$^{19}$-$X$-$X$-Asp$^{22}$ $-$ sequence. In the three crystal structures of the K24M and K31M/K32M mutant proteins and peptide 1 forms of IA3 complexed with proteinase A, the $\epsilon$-NH$_2$ group of Lys18 of the inhibitor hydrogen bonds to one of the carboxyl oxygens of Asp28 of the enzyme (Fig. 3). This is one of the two catalytic Asp residues that operate the catalytic mechanism of all aspartic proteinases (24). The $\epsilon$-NH$_2$ group of Lys18 also hydrogen bonds to one of the carboxyl oxygens of the side chain of Asp22 in the IA3 inhibitory sequence (Fig. 3). The other oxygen of the side chain COOH of Asp22 hydrogen bonds to the phenolic OH group of Tyr75 in the enzyme, a residue that is totally conserved in all eukaryotic aspartic proteinases and which is positioned almost at the tip of the $\beta$-hairpin loop or flap that overlays the active site cleft in these enzymes. A network of interactions thus cross-links these charged residues of IA3 with the catalytically...
essential and structurally conserved residues of the target enzyme (Fig. 3). When the charged side chain of Asp$^{22}$ in the full-length protein form of IA$_3$ was replaced with the hydrophobic but otherwise almost isosteric side chain of a leucine residue, the purified D22L mutant protein had a slightly reduced potency both at pH 3.1 and 4.7 (Table I) but nevertheless was still an effective inhibitor.

However, when Lys$^{18}$ was changed to Met in concert with the D22L mutation, the resultant inhibitor (K18M/D22L, Table I) was an extremely potent inhibitor. For the first time in all of these studies, it was not possible to derive an accurate $K_c$ value at pH 3.1 because the protein-protein interaction was so tight. This may be a reflection of the increased propensity of Met and Leu residues to be accommodated within a helical conformation by comparison with their wild-type Lys and Asp counterparts. Alternatively, this may be a further indication of the importance of hydrophobic contributions to binding strength. Modeling studies suggest that the side chain of a methionine at position 18 in the IA$_3$ sequence is surrounded by the hydrophobic side chains of Ile$^{20}$, Tyr$^{27}$, Thr$^{111}$, Phe$^{112}$, Phe$^{117}$, and Ile$^{120}$ of proteinase A, as well as the newly introduced side chain of Leu$^{22}$ in the K18M/D22L double mutant inhibitor. The side chain of Leu$^{22}$ can make potential, favorable interactions with C-β of Ser$^{35}$ and the side chains of Ile$^{73}$ and Tyr$^{75}$ from the flap of proteinase A, as well as with the Met$^{18}$ and Val$^{25}$ residues of the IA$_3$ inhibitory sequence.

The importance of hydrophobic interactions was further corroborated when the Leu$^{18}$ residue within the $\sim$Lys$^{18}$-Leu$^{19}$-X-X-Asp$^{22}$-centerpiece was changed to Ala in the peptide form of IA$_3$. The resultant peptide (peptide 27, Table IV) was no longer an inhibitor at pH 3.1 and an apparent inhibition constant of 700 nM was estimated at pH 4.7. This loss in potency (compare peptides 27 and 9, Table IV) was the largest observed for any single amino acid replacement. However, when peptide 27 was incubated with proteinase A for 16 h at pH 4.7 and 37 °C at a molar ratio of 40:1, it was cleaved as a substrate (as monitored by reverse phase fast protein liquid chromatography, not shown). Peptide 27 is thus a good alternative substrate at pH 4.7 and was giving only an apparent inhibition of the activity of proteinase A toward the chromogenic substrate used in the inhibition assays. The deletion of the terminal isopropyl moiety of the leucine side chain thus converted a highly potent polypeptide inhibitor into a regular substrate of proteinase A by decreasing affinity to the target enzyme and possibly by reducing internal helix stability.

Under the same conditions, peptides 14 and 8 (Table II) which together span the 2–34 sequence of IA$_3$ were both cleaved by proteinase A (at the $\sim$Glu$^{10}$-Ile$^{11}$- and $\sim$Ala$^{29}$-$^{39}$ bonds, respectively) as reported previously (12). Peptide 7, despite having been synthesized deliberately to position a valine bond was no longer an option, it was still cleaved albeit very slowly (50% in 16 h) upon incubation at pH 4.7 with proteinase A at a molar ratio of 40:1. Since cleavage at the mutated $\sim$Val$^{29}$-$^{30}$ bond was no longer an option, it was found by amino acid analysis that processing had taken place at the adjacent $\sim$Phe$^{30}$-$^{31}$ bond (data not shown). Similarly, peptides 12 and 13 (Table II) were digested after 72 h incubation with proteinase A at a molar ratio of 10:1, as were peptides 18, 19, 20, and 26 of the peptides listed in Table IV. Thus, in addition to the L19A mutant (peptide 27) as described above, the other peptides (7, 8, 12, 13, 14 (Table II), 18, 19, 20, and 26 (Table IV)) which were not effective as inhibitors of proteinase A, served instead as substrates for the enzyme.

From all of these data, it is readily apparent that the inhibitory capacity of IA$_3$ is located within residues 2–34 of the sequence and that these residues need to be present as a contiguous polypeptide to achieve inhibition. The inhibitory sequence of residues is not cleaved by the target enzyme but only very subtle alterations in sequence are necessary to convert the IA$_3$ polypeptide into a substrate for proteinase A. In contrast, however, the wild-type polypeptide is readily hydrolyzed as a substrate by nontarget aspartic proteinases such as pepsin and cathepsin D, which have substantial similarity to proteinase A in their primary sequences and three-dimensional structures (25–27). This substantiates our NMR and CD findings reported previously (12) that free IA$_3$ in its peptide or protein forms is predominantly unstructured. The interaction of IA$_3$ with its target enzyme appears to depend critically on the insertion of three hydrophobic “pins” (the front-end cluster, Leu$^{19}$ and the back end cluster) into the appropriate hydrophobic sockets provided by the active site cleft of proteinase A. The target enzyme thus plays the role of a helper template, stabilizing the amphipathic helical conformation of IA$_3$ but, paradoxically, resulting in its own inhibition. In contrast, the random coil IA$_3$ polypeptide is apparently unable to locate the critical pins sufficiently precisely in its interaction with the nontarget aspartic proteinases. Consequently, since aspartic proteinases generally require only 7 or 8 amino acid residues of a polypeptide to bind in an extended β-strand conformation in their active site to act as a substrate, the nontarget enzymes are readily able to cleave IA$_3$. Thus, the “default” setting is cleavage of the random coil IA$_3$ polypeptide. In contrast, helicidation requires many more stringent conditions to be fulfilled such as complementary amphipathicity, precision of shape, and the correct juxtaposition of side chains possessing the appropriate properties to fit snugly into the hydrophobic pockets of proteinase A. Only if IA$_3$ can be sculpted in situ to become the key that matches precisely into the lock that is the active site of proteinase A, can the IA$_3$ polypeptide escape cleavage and preserve its integrity. Thus, in its interaction with this aspartic proteinase, IA$_3$ is stabilized in the somewhat abnormal helical conformation, shaped by the proteinase itself but yet at the same time, held clear of the catalytic machinery through formation of the helix. The alternative, as a consequence of any imprecision of fit, is recognition in an extended conformation and cleavage.

The present study has thus afforded considerable insight into the features that are important in governing the potency and selectivity of inhibition of proteinase A by IA$_3$. This presents a fascinating challenge for future investigation to determine whether this unprecedented mode of inhibitor-enzyme interaction can be exploited to generate selective inhibitors re-targeted against other aspartic proteinases including those produced by pathogenic organisms.

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The Potency and Specificity of the Interaction between the IA₃ Inhibitor and Its Target Aspartic Proteinase from *Saccharomyces cerevisiae*

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