Key Features Determining the Specificity of Aspartic Proteinase Inhibition by the Helix-forming IA3 Polypeptide

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The 68-residue IA3 polypeptide from Saccharomyces cerevisiae is essentially unstructured. It inhibits its target aspartic proteinase through an unprecedented mechanism whereby residues 2–32 of the polypeptide adopt an amphipathic α-helical conformation upon contact with the active site of the enzyme. This potent inhibitor (K<sub>i</sub> < 0.1 nM) appears to be specific for a single target proteinase, saccharopepsin. Mutagenesis of IA3 from S. cerevisiae and its ortholog from Saccharomyces castellii was coupled with quantitation of the interaction for each mutant polypeptide with saccharopepsin and closely related aspartic proteinases from Pichia pastoris and Aspergillus fumigatus. This identified the charged K18/D22 residues on the otherwise hydrophobic face of the amphipathic helix as key selectivity-determining residues within the inhibitor and implicated certain residues within saccharopepsin as being potentially crucial. Mutation of these amino acids established Ala-213 as the dominant specificity-governing feature in the proteinase. The side chain of Ala-213 in conjunction with valine 26 of the inhibitor marshals Tyr-189 of the enzyme precisely into a position in which its side-chain hydroxyl is interconnected via a series of water-mediated contacts to the key K18/D22 residues of the inhibitor. This extensive hydrogen bond network also connects K18/D22 directly to the catalytic Asp-32 and Tyr-75 residues of the enzyme, thus deadlocking the inhibitor in position. In most other aspartic proteinases, the amino acid at position 213 is a larger hydrophobic residue that prohibits this precise juxtaposition of residues and eliminates these enzymes as targets of IA3. The exquisite specificity exhibited by this inhibitor in its interaction with its cognate folding partner proteinase can thus be readily explained.

Aspartic proteinases are widely distributed in nature and are involved in a variety of physiological processes and pathological conditions such as Alzheimer disease, cancer, hypertension, and AIDS (1). In stark contrast, naturally occurring protein inhibitors of aspartic proteinases are rare and are found only in a few specialized locations (1–3). There is thus a need to understand the mechanism of action of the few inhibitors that do exist with a view to exploitation of their therapeutic potential. One such inhibitor is the IA3 polypeptide from Saccharomyces cerevisiae. This is a potent inhibitor (K<sub>i</sub> < 10<sup>-10</sup> M) of its target enzyme, the vacuolar proteinase from S. cerevisiae, known previously as proteinase A but which is now better denoted as saccharopepsin (2). Remarkably, IA3 appears to be completely specific for this sole target proteinase (3). A wide variety of other aspartic proteinases of vertebrate, parasite, plant, and fungal origin are not only unaffected by IA3 but have been shown to digest it as a substrate (3, 4).

Although IA3 consists of 68 residues, we have shown previously that all of the inhibitory activity resides within the N-terminal half of the polypeptide (3). By itself, free IA3 is essentially unstructured (5), but, upon contact with its target proteinase, the polypeptide operates as an inhibitor through an unprecedented mechanism (3, 4). Residues 2–32 become ordered and adopt an almost perfect amphipathic helical conformation occupying the active site of the enzyme (3, 4). The hydrophilic face of the IA3 helix is oriented predominantly toward the solvent, whereas the face enclosed by the target enzyme is largely hydrophobic. Our previous biochemical and crystallographic investigations have revealed that the potency of the interaction of IA3 with saccharopepsin is mostly generated by the insertion of a series of hydrophobic amino acids into complementary hydrophobic pockets provided by the active site cleft of the enzyme (3, 4). Within the IA3 inhibitory sequence of residues 2–34 (Fig. 1), these consist of (i) an N-terminal cluster of V8–X–X–I11–F12, (ii) a C-terminal cluster of V26–X–X–A29–F30, and (iii) the leucine residue at position 19. This is found within the sequence K18-L19-X–X–D22, which also contributes, somewhat unexpectedly, two charged amino acids, K18 and D22, in an i, i+4 pair, to the hydrophobic face of the amphipathic helical inhibitor. The ε-NH<sub>2</sub> group of the K18 side chain is held in place through three hydrogen bonds, including one with the carboxyl group of Asp-32 from the enzyme and another with one of the carboxyl oxygens of the side chain of D22 (3, 4). The other carboxyl oxygen of D22 is hydrogen-bonded to Tyr-75 contributed by the β-hairpin loop or “flap” that overlies the
active site of the enzyme (4, 6). Through this intricate network of strong hydrogen bonds, K18 and D22 are tightly linked to each other and to crucial residues (Asp-32 and Tyr-75) of the enzyme’s active site (3, 4). However, since Asp-32 and Tyr-75 are totally conserved in the sequences of all active aspartic proteinases from eukaryotic sources, this arrangement cannot by itself account for the very high specificity observed in the interaction between IA3 and its cognate folding partner proteinase, saccharopepsin. Nevertheless, the K18 and D22 residues in the inhibitor were selected as the starting point for our investigations to identify the key features that facilitate this selective interaction because of their unusual positioning on the hydrophobic face of the helix, where they contribute little to the energetics of inhibitor binding. This was demonstrated recently by varying these residues considerably without diminishing the potency of the IA3 interaction with saccharopepsin at the standard pH of 4.7 employed throughout our studies (6). Their influence on selectivity was established initially by mutagenesis of these residues and determination of the resultant effects on the inhibition not only of saccharopepsin but also of two fungal vacuolar aspartic proteinases (from Pichia pastoris and Aspergillus fumigatus) that are closely related to saccharopepsin (sequence identity ~75%). Inspection of x-ray structures of IA3-inhibited saccharopepsin (3, 4) and models of these two other fungal proteinases identified, in turn, potential key residues for mutation within the saccharopepsin component of this remarkable proteinase/inhibitor partnership.

EXPERIMENTAL PROCEDURES

DNA Manipulation and Production of Recombinant Proteinases—Mutations were introduced by overlapping PCR mutagenesis (7) into the wild-type saccharopepsin sequence to generate clones encoding the Thr222Ala, Thr287Met/pro288Gly, and Ala213Ile mutant proteinases. The respective proteinases were selected as the starting point for our investigations to identify the key features that facilitate this selective interaction because of their unusual positioning on the hydrophobic face of the helix, where they contribute little to the energetics of inhibitor binding. This was demonstrated recently by varying these residues considerably without diminishing the potency of the IA3 interaction with saccharopepsin at the standard pH of 4.7 employed throughout our studies (6). Their influence on selectivity was established initially by mutagenesis of these residues and determination of the resultant effects on the inhibition not only of saccharopepsin but also of two fungal vacuolar aspartic proteinases (from Pichia pastoris and Aspergillus fumigatus) that are closely related to saccharopepsin (sequence identity ~75%). Inspection of x-ray structures of IA3-inhibited saccharopepsin (3, 4) and models of these two other fungal proteinases identified, in turn, potential key residues for mutation within the saccharopepsin component of this remarkable proteinase/inhibitor partnership.

DNA Manipulations and Production of Recombinant Inhibitors—Mutations were introduced, simultaneously where appropriate, at residues 18 and 22 to produce some mutant forms of S. cerevisiae IA3 (inhibitors 2–11, Tables 2 and 3), as described previously (6). The QuikChange kit (Stratagene) or overlapping PCR methods (7) were used to introduce the mutations, using the primer pairs as detailed in supplemental Table 1. Other mutant inhibitors (inhibitors 16–18 described in Table 4 and 25–27 in Table 6) were produced by engineering a cassette version of the DNA encoding S. cerevisiae IA3. An unwanted Sacl site near the 3’-end of the IA3 sequence was removed, and an Nhel site was introduced as a silent mutation in the codons for Ala-34-Ser-35 (GCT AGT → GCT AGC) by (separate) site-directed mutageneses using QuikChange. Digestion with Sacl/Nhel enabled removal of the bases encoding wild-type residues 15–34. Replacement with appropriate pairs of synthetic oligonucleotides carrying the desired base changes enabled the mutations to be introduced; the oligonucleotide pairs used for these mutant forms of IA3 are shown in supplemental Table 1.

Genomic DNA was extracted from S. castellii, and the gene encoding the inhibitor ortholog was amplified by PCR using 5’-CATATGAGTGATCTAAACGCCGCTACGGT-3’ and 5’-GGTCGACCTAATAGACCTTGCTAAAACCTAC-3’ as forward and reverse primers, respectively. Following cloning into the vector used for wild-type saccharopepsin, expression was performed in Saccharomyces cerevisiae cells deficient in saccharopepsin (8). The recombinant Pichia proteinase was purified from the conditioned medium using the same protocol as that described previously for saccharopepsin (3, 4). Sequencing (through 12 cycles of Edman degradation) of the recombinant Pichia proteinase gave Ala-Ser-His-Asp-Ala-Pro-Leu-Thr-Asn-Tyr-Leu-Asn–, which corresponds precisely to that of the mature form of the proteinase predicted by the DNA sequence.

A clone encoding the precursor of the vacuolar aspartic proteinase from A. fumigatus in the pPICZαA vector was a kind gift from Professor M. Monod (Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland). This was expressed in P. pastoris cells as described in Ref. 9, and the recombinant proteinase was purified to homogeneity from the culture medium by a slight modification of the protocol originally described (9). This was necessary to remove the yellow pigment that is produced in conditioned medium produced by P. pastoris cells. Briefly, this involved dialysis of the culture medium for 72 h against 10 mM sodium formate buffer, pH 4.0, containing 1 mM EDTA, followed by successive concentrations in a stirred cell concentrator (Vivascience Sartorius Ltd., Epsom, UK) fitted with a 5-kDa cut-off membrane. The concentrate was subjected to successive chromatographies on Sephadex G-50 and a Resource Mono S column (Amersham Biosciences) in the same formate buffer. Elution from the ion exchange column was achieved with a 0–50% gradient of the formate buffer containing 1 mM NaCl. The purified Aspergillus enzyme was found to have microheterogeneity at its N terminus, as described in the original report on the production of this recombinant enzyme (9).

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duced into this wild-type sequence to generate the M18K/K22M, M18K/K22L, and M18K/K22D variants (inhibitors 13–15 respectively, Table 3) using the primers and methods indicated in supplemental Table 1.

Wild-type and mutant forms of IA3 from *S. cerevisiae* and *S. castellii* were subcloned into the NdeI-XhoI sites of pET-22b (Novagen, Milton Keynes, UK), thus introducing a C-terminal Leu-Glu-His, tag as described previously (3, 4, 6). All recombinant protein forms were produced in *Escherichia coli* and purified to homogeneity by nickel-chelate chromatography, as reported previously (3, 4, 6). Synthetic peptide forms (inhibitors 19–24) consisting of residues 2–32 or 2–34 from the *S. cerevisiae* or *S. castellii* sequences (Fig. 1) were obtained from Alta Biosciences (Birmingham, UK) and had L-norleucine residues introduced in place of methionine, where appropriate, as discussed previously (3, 4).

Inhibition assays were carried out predominantly at pH 4.7, but in some cases, when the *K* value at this pH lay at or beyond the limit of accurate determination using the assay methodology available, it was necessary to make measurements at an alternative pH; the lower pH of 3.1 was used in these instances, as described previously (3, 4, 6). The synthetic chromogenic substrate (from Alta Biosciences) used in all assays was Lys-Pro-Ile-Glu-**Nph**-Arg-Leu (where the asterisk represents the scissile peptide bond, and Nph represents L-nitrophenylalanine (3, 4, 6)). N-terminal sequencing was performed by automated Edman degradation (Alta Bioscience). Modeling calculations were carried out on an SGI Octane workstation with dual R12000 processors, using the Moloc program, as reported previously (3, 6).

**RESULTS**

*Interaction of Variant Forms of IA3 with Fungal Proteinases—*

Two fungal aspartic proteinases that are closely related to saccharopepsin were identified by searching the sequence data bases. These enzymes, from *P. pastoris* (PpPr) and *A. fumigatus* (AfPr), have 77 and 71% identity, respectively, with the overall sequence of saccharopepsin and, in this regard, are more closely related to saccharopepsin than any of the aspartic proteinases we had tested and in combination, were carried out. Against PpPr, introduction of only one hydrophobic residue (M at position 22) resulted in a marginal increase in inhibitory potency (cf. 5 in

| Proteinase               | *Km* cat | *kcat* | ki cat | Km cat |
|--------------------------|----------|--------|--------|--------|
| *P. pastoris*            | 0.06     | 45     | 7 × 10^2 |        |
| *A. fumigatus*           | 0.19     | 66     | 3 × 10^8 |        |
| Wild-type saccharopepsin | 0.14     | 44     | 3 × 10^7 |        |
| Thr222Ala saccharopepsin | 0.10     | 21     | 2 × 10^6 |        |
| Thr227Met/Pro288Gly saccharopepsin | 0.12 | 21 | 2 × 10^6 |        |
| Ala213lle saccharopepsin | 0.25     | 66     | 3 × 10^7 |        |

*Specificity of IA3 Inhibition*

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The M18/K22 Combination Occurs Naturally—In order to expand our study further, data bases were searched repeatedly for sequences resembling that of IA3. The only significant hits that were obtained were from yeast species closely related to S. cerevisiae. The five new orthologs thus identified are aligned in Fig. 1. Four of these new IA3-like proteins (from Saccharomyces bayanus, Saccharomyces paradoxa, Saccharomyces kudriavzeii, and Saccharomyces mikatae) were almost identical to S. cerevisiae IA3 in the inhibitory sequence region (residues 2–34) with one, two, three, and three changes respectively, all in non-critical residues. In contrast, the sequences of residues 36–53 were highly variable, implying that this region is indeed unimportant for inhibitory function. Indirectly, this also substantiates our previous experimental demonstration that the inhibitory activity is located within the N-terminal half of the IA3 polypeptide (3, 4). However, the fifth sequence, from S. castellii, displayed a mere 45% sequence identity with S. cerevisiae IA3 (residues 2–34) and most notably had an M18/K22 combination of hydrophobic/positively charged residues as its wild-type arrangement (Fig. 1). In addition, with 81 residues overall, it was considerably longer than the other IA3 sequences.

Since the S. castellii protein offered a variant with considerably different sequence, it was produced in recombinant form in E. coli and purified to homogeneity as described above for the S. cerevisiae IA3 inhibitors. This M18/K22-containing wild-type protein was effective as an inhibitor of saccharopepsin, albeit with a potency that was reduced by ~20-fold relative to the K18M/D22K double mutant IA3 from S. cerevisiae (cf. 12 and 11 in Table 3). A small (5-fold) reduction in potency against PpPr was similarly observed for the same pair of inhibitors. Taken together with a comparison of the sequences of the S. castellii and S. cerevisiae polypeptides (Fig. 1), this establishes that more than 50% of residues 2–34 can be substituted without dramatic effects on inhibition and suggests that a few key amino acids must be of the correct nature in order to achieve potent inhibition.

This was substantiated by reversal of the wild-type M18/K22 arrangement in the S. castellii sequence to K18/M22 or mutation to K18/L22. These alterations essentially destroyed the ability to inhibit both saccharopepsin and PpPr (13 and 14 in Table 3). However, when the endogenous M18/K22 combination in the S. castellii sequence was replaced by the K18/D22
pairing of \textit{S. cerevisiae} IA$_3$, the resultant inhibitor (15 in Table 3) showed comparable potency to the wild-type \textit{S. castellii} protein (12 in Table 3) against saccharopepsin and, separately, against PpPr. Thus, within the context of the \textit{S. castellii} sequence, the K/D and M/K combinations of residues at positions 18 and 22 are equally effective in promoting inhibition of saccharopepsin and, separately, PpPr. By contrast, within the \textit{S. cerevisiae} sequence context, whereas the mutant M18/K22 combination results in a small loss in inhibitory potency against saccharopepsin relative to the wild-type K18/D22 pairing, it produces a 20-fold improvement in potency over the K18/D22 pairing toward PpPr (cf. 11 in Table 3 with 1 in Table 1). In addition to this comparison of the potency of pairs of inhibitors against individual enzymes, it is also informative to examine the abilities of each inhibitor to interact with the two proteinases PpPr and saccharopepsin. The ratio of $K_i$ values for the wild-type M18/K22-containing \textit{S. castellii} protein against the two enzymes is 4 (12 in Table 3). The same ratio was derived for the K18/D22 mutant \textit{S. castellii} protein (15 in Table 3), and a value of 15, comparable in magnitude, was obtained for the M18/K22 mutant form of \textit{S. cerevisiae} IA$_3$ (inhibitor 11). In contrast, the wild-type K18/D22-containing IA$_3$ from \textit{S. cerevisiae} is more than 550 times more effective as an inhibitor of saccharopepsin than of PpPr (1 in Table 2). Thus, in order to be effectively inhibited by \textit{S. cerevisiae} IA$_3$, with its positively charged K18 accompanied by D22, it would appear that hydrogen bond receptors/donors must be arranged in precisely the positions required to form a network of hydrogen bonds that satisfies all the donors/acceptors of these two amino acids. Lengthening or breaking hydrogen bonds is costly in energy terms, so a small change in inhibitor position might be expected to have a negative effect on attractive hydrogen bond interactions.

In order to establish whether the 18/22 pair of residues was the predominant factor influencing selectivity, other residues from the \textit{S. castellii} polypeptide sequence were introduced increasingly into \textit{S. cerevisiae} IA$_3$. Replacement of the V26-X-X-A29-F30 residues in the C-terminal hydrophobic cluster (see Introduction) by the counterpart residues from the \textit{S. castellii} sequence produced inhibitor 16 (Table 4). This was such a potent inhibitor of saccharopepsin as to preclude accurate quantitation of its $K_i$ value. However, the ratio of $K_i$ values for PpPr/saccharopepsin remained a large number (Table 4), and a comparable effect was obtained for inhibitor 17 with an additional Q20E substitution. However, when only the M18 and K22 residues from the \textit{S. castellii} sequence were introduced into \textit{S. cerevisiae} IA$_3$, the lower value of 15 was observed for the $K_i$ ratio (11 in Table 4), as discussed above. Furthermore, when all 10 of the residues in positions 18–32 of \textit{S. cerevisiae} IA$_3$ that differ from those in the \textit{S. castellii} sequence were replaced by their \textit{S. castellii} counterparts, the resultant chimeric inhibitor 18 was almost as potent toward PpPr as against saccharopepsin (Table 4). When the reciprocal C-terminal cluster residues from \textit{S. cerevisiae} IA$_3$ were introduced, singly and in combination, into the (M18/K22-containing) \textit{S. castellii} sequence, the resultant inhibitors also displayed ratios of $K_i$ values against PpPr/saccharopepsin that varied between 2 and 5 (data not shown).

Thus, with an M/K combination present at 18/22, the sequences can inhibit PpPr almost as well as saccharopepsin, with the other residues between 18 and 32 appearing to have only a minor influence on the selectivity of inhibition. By contrast, with K/D at 18/22, although the interaction with saccha-
Roepepsin is slightly improved, the inhibition of PpPr is very significantly disfavored.

Structural Differences among the Three Fungal Proteinases—

These investigations established the dominant influence of the residues at positions 18 and 22 in the S. cerevisiae IA3 sequence in determining whether an enzyme other than saccharopepsin could be inhibited. However, these manipulations resulted in inhibitory activity only against the Pichia proteinase. None of the inhibitors described above (5–18 in Tables 3 and 4) showed any significant inhibitory effect against AfPr. Thus, the differences in sequence between AfPr and saccharopepsin would appear to be more likely to engender resistance to inhibition than those differences between PpPr and saccharopepsin. Insufficient amounts of PpPr were generated for crystallographic purposes, so models were generated for the PpPr and AfPr enzymes based on the several crystal structures that we have described previously for saccharopepsin complexed with (variant) IA3 molecules from S. cerevisiae (3, 4). With ~75% identity in sequence, the three proteinases inevitably have essentially the same three-dimensional structure overall. Of the enzyme residues that make direct contact with S. cerevisiae IA3 in the crystal structure(s), virtually all are identical in saccharopepsin, AfPr, and PpPr.

One exception is at residue 295, where PpPr has Ile in place of Val in saccharopepsin and AfPr (Fig. 2). This residue is located in a region of the sequence from residue 290 to 300, part of which influences binding of the S2' and S3' residues of a substrate and is known as the "polyproline loop" in aspartic proteinases (10). However, AfPr and saccharopepsin have identical sequences between residues 289 and 300. Since AfPr is much more resistant to inhibition than PpPr as described earlier, the Val295Ile change would not appear to be a dominant factor in determining susceptibility to inhibition and so was not investigated further. The only other direct contact residue that differs among the three fungal vacuolar proteinases is residue 222 (Fig. 2). This is one of the main contributors that shapes the binding pocket for the crucial L19 residue of IA3 as reported previously (3, 4). Residue 222 (Thr in saccharopepsin but Ala in PpPr and AfPr) sits between residues 287 and 300 in a linear arrangement (Fig. 3A). In turn, residue 300 sandwiches residue 213 between itself and residue 189. Whereas residues 300 and 189 in this arrangement are conserved in identity among the aspartic proteinases being considered here, residues 287 and 213 differ. Although the latter two do not make direct contact with the IA3 inhibitor, their involvement in and proximity to the other residues depicted in the arrangement in Fig. 3A was considered to

![Figure 3A](image-url)

**FIGURE 3A.** Superposition of selected residues from saccharopepsin in its uninhibited (pink; Protein Data Bank code IFMU) and IA3-complexed (blue; Protein Data Bank code 1DPJ) forms. The IA3 inhibitor with the side chains of V26 (in space fill) and L19 is shown in yellow. B, space filling representation of the side chains of (left to right) Tyr-189, Ala-213 (green), Ile-300, and Thr-222 in saccharopepsin, with the IA3 inhibitor and its residue at position 26 displayed in yellow. C, connections between the side chains of the key K18/D22 feature of IA3 (yellow) and the key Ala-213 residue (green) contributed by saccharopepsin. A network of hydrogen bonds connects K18 and D22 to Asp-32, Asp-215, Tyr-75, and Tyr-189 of the enzyme via a series of water molecules 1–5. Water 2 is known as the catalytic water molecule and is present in all aspartic proteinases (1, 4, 11).
be of potential importance. Consequently, mutations were introduced into saccharopepsin to replace the wild-type residues present at the “variable” positions of 213, 222, and 287 (Fig. 2). In the Thr-287 case, however, the substitution with Met was actually introduced as a double mutation, Thr287Met/Pro288Gly, because the relatively “stiff” Pro-288 residue that is present in saccharopepsin and PpPr is a much more flexible Gly residue in AfPr. This was considered to have a possible influence on the ease of deformability of the polyproline loop (which immediately follows residue 288 in the sequence (Fig. 2)) and hence the adaptability of the loop in its interaction with the helical inhibitor.

**Mutant Forms of Saccharopepsin**—The three mutated variants of saccharopepsin, Thr222Ala, Thr287Met/Pro288Gly, and Ala213Ile, were produced as described under “Experimental Procedures.” In all three cases, the mutants exhibited kinetic parameters comparable with those of wild-type saccharopepsin for cleavage of our synthetic peptide substrate (Table 1) and were just as susceptible to inhibition by the universal aspartic proteinase inhibitor, pepstatin (data not shown). In addition, the $K_i$ values determined for the interactions of the Thr222Ala and Thr287Met/Pro288Gly mutants with the full-length wild-type *S. cerevisiae* IA3 remained so tight as to lie beyond the limits of accurate determination (<0.1 nm). Clearly, this precluded distinction between changes in potency. We have, however, reported previously that shortening the length of the *S. cerevisiae* inhibitor weakens its potency against wild-type saccharopepsin (3). Consequently, a peptide consisting only of residues 2–32 of the *S. cerevisiae* IA3 sequence was tested in wild-type form (inhibitor 19) and as a variant with a bulker phenylglycine substituent at position 19 (inhibitor 20). For both inhibitors, the $K_i$ values determined for the Thr222Ala mutant enzyme were closely similar to those derived for wild-type saccharopepsin (19 and 20 in Table 5). Thus, the identity of the enzyme residue at position 222 (Thr or Ala) helps to sculpt the binding pocket for residue 19 of IA3, and this appears to have little influence on the ability of IA3 to interact with its folding template partner. Similarly, the $K_i$ values measured for inhibitors 19 and 20 interacting with the Thr287Met/Pro288Gly mutant enzyme were only modestly altered (by ~4-fold) relative to those of the wild-type enzyme (Table 5).

For the Ala213Ile mutant saccharopepsin, however, the $K_i$ value measured for the polypeptide consisting of residues 2–34 of *S. cerevisiae* IA3 had worsened by several hundred-fold relative to the value for wild-type saccharopepsin (21 in Table 6). A version of this sequence that was shorter in length and so had a readily quantifiable $K_i$ value against wild-type saccharopepsin was also much less potent (by more than 2 orders of magnitude) against the Ala213Ile mutant enzyme (22 in Table 6).

A number of chimeric inhibitors were then examined in which residues from the *S. castellii* sequence that differ from those in *S. cerevisiae* IA3 were introduced into the sequence of the latter. In inhibitor 23, 11 residues (including M18/K22) between positions 2 and 22 were thus derived from the *S. castellii* sequence. This chimeric inhibitor was 35-fold less effective against the Ala213Ile mutant saccharopepsin (Table 6). Inhibitor 24 corresponded to the entire sequence of residues 2–34 from *S. castellii* and was, once again, 35-fold less effective against the Ala213Ile mutant enzyme compared with wild-type saccharopepsin (Table 6). When only the M18/K22 combination of residues from *S. castellii* was introduced into the sequence of the *S. cerevisiae* IA3, the resulting inhibitor was 35-fold less effective against the Ala213Ile mutant (Table 6).

**TABLE 5**

| Identify | Residue 19 | Wild type | Thr222Ala | Thr287Met/Pro288Gly |
|---------|-----------|-----------|-----------|---------------------|
|          |           |           |           |                     |
| 19       | Leu       | 2 ± 0.5   | 4 ± 0.5   | 8 ± 1               |
| 20       | Phenylglycine | 9 ± 1     | 14 ± 1    | 35 ± 6              |

**TABLE 6**

| Identifier | Sequence | Wild-type $K_i$ (nM) | Ala213Ile $K_i$ (nM) | Ratio |
|------------|----------|----------------------|---------------------|-------|
| 21         | NTOQ QKVS IFQSS KEKLQ GDAKV VSADF KKA | ≤0.1 | 60 ± 7 | >600 |
| 22         | NTOQ QKVS IFQSS KEKLQ GDAKV VSADF KK | 6 ± 1 | 930 ± 120 | 155 |
| 23         | SDRN ANVS ZFQA KELEL GDAKV VSADF KKA | 0.8 ± 0.1 | 30 ± 2 | 35 |
| 24         | SDRN ANVS ZFQA KELEL GRANA ASELG KNSA | 0.4 ± 0.1 | 15 ± 2 | 35 |
| 11         | NTOQ QKVS IFQSS KEMLQ GDAKL VSADF KKN | 0.2 ± 0.04 | 6 ± 1 | 30 |
| 25         | NTOQ QKVS IFQSS KMLE GRANA VSDFG KNSA | 4 ± 1 | 65 ± 4 | 15 |
| 26         | NTOQ QKVS IFQSS KMLE GRANA VSBMG KNSA | 7 ± 2 | 110 ± 10 | 15 |
| 18         | NTOQ QKVS IFQSS KMLE GRANA ASEGM KNSA | 2 ± 0.3 | 14 ± 2 | 7 |
| 27         | NTOQ QKVS IFQSS KEKLQ GDAKL ASGGM KNSA | <0.1 | 30 ± 8 | >300 |
introduced in place of the wild-type K18/D22 residues in the *S. cerevisiae* IA₃ sequence, the loss in potency against the Ala213lle mutant saccharopepsin remained at around 30-fold (inhibitor 11, Table 6). Progressive introduction into inhibitor 11 of further *S. castellii* residues at positions 25, 26, 29, and 30 resulted in inhibitors that showed potencies against the Ala213lle mutant saccharopepsin that were only 15-fold weaker than those measured for the wild-type enzyme (inhibitors 25 and 26, Table 6). For inhibitor 18, which contained the full complement of *S. castellii* residues at positions 18/22, 25, 26, 29, and 30, only a 7-fold difference was measured in the *Kᵢ* values obtained for the Ala213lle mutant and wild-type enzymes (Table 6). This same inhibitor 18 was shown earlier to have essentially the same potency for wild-type saccharopepsin and the *Pichia* proteinase (Table 3). By contrast, combination of the *S. castellii* residues at positions 25, 26, 29, and 30 with K/D at positions 18/22 resulted in a ratio of *Kᵢ* values for the Ala213lle mutant and wild-type saccharopepsins that was more than 300 (27 in Table 6). Thus, with a K18/D22 pairing present in the inhibitory sequence, large differences are observed in the *Kᵢ* values when isoleucine is introduced in place of the wild-type alanine 213 in saccharopepsin (inhibitors 21, 22, and 27, Table 6). This distinction is considerably smaller when inhibitors have the M18/K22 combination (inhibitors 11, 18, and 23–26, Table 6). In all cases, variation within residues 2–32 at positions other than 18 and 22 appears to have relatively minor effects.

**DISCUSSION**

*Key Feature in the Proteinase Partner*—These mutation studies have identified that the dominant features in the proteinase/inhibitor couple that combine to restrict this protein/protein interaction are (i) residue 213 in the enzyme and (ii) the K18/D22 pairing on the otherwise hydrophobic face of the *S. cerevisiae* inhibitor. From a survey of the sequences of 850 aspartic proteinases from a diversity of species (available on the World Wide Web at www.sanger.ac.uk/software/pfam), the residue at position 213 is isoleucine in 63% of cases. Together with leucine (16%) and valine (11%), these three amino acids with large side chains account for an overwhelming ~90% of the occupancy at this position. In contrast, a smaller Ala residue, as in saccharopepsin, is found in only 3.6% of the sequences. Thus, by this simple expedient, IA₃ is able to eliminate a large number of aspartic proteinases as effective targets. The Ala213lle mutant saccharopepsin was not produced in sufficient amounts to make crystallization attempts of its complexes with any of these inhibitors a realistic possibility. Nevertheless, examination of the crystal structures that we have reported previously for wild-type saccharopepsin complexed with variant IA₃ sequences (3, 4) reveals that Ala-213 is located snugly in between Ile-300 and Tyr-189 (Fig. 3B). In the IA₃ inhibited complexes, the Tyr-189 side chain is significantly relocated relative to its position in the structure solved for uninhibited saccharopepsin (Protein Data Bank code IFMU) (11) as a consequence of its proximity to the side chain of residue 26 from the inhibitor (Fig. 3A). In the case of wild-type saccharopepsin with the small side chain of Ala at position 213, this is sterically tolerated. However, with a more voluminous isoleucine at position 213, Tyr-189 would be prevented from undergoing this side chain reorientation. During the helicating process of IA₃, repositioning of this tyrosine side chain may lead to a more severe steric clash with the inhibitor residue at position 26. Thus, the presence of an alanine at position 213 in combination with residues in less crucial locations (e.g. Thr-222 and Thr-287-Pro-288) makes a major contribution from the enzyme to the exquisite specificity that is observed in the interaction with IA₃.

*Key Feature in the Inhibitor Partner*—Inhibitor 24, which consists solely of residues 2–34 from the *S. castellii* sequence, was found to be a subnanomolar inhibitor of wild-type saccharopepsin from *S. cerevisiae* (Table 6). This finding establishes clearly that the inhibitory activity of the *S. castellii* polypeptide is located in the N-terminal 34 residues of the molecule, just as was reported previously for *S. cerevisiae* IA₃ (3, 4). In the sequences (residues 2–34) of the *S. castellii* and *S. cerevisiae* polypeptides, most of the residues that differ are hydrophilic and are oriented toward solvent, making no significant contacts with the proteinase. Although each of the residues (A25, A26, G29, and M30) contributing the C-terminal hydrophobic cluster in the *S. castellii* sequence is smaller than its counterpart (V25, V26, A29, and F30) in *S. cerevisiae* IA₃, perhaps the most noteworthy difference occurs in the i, i + 4 pairing of positions 18/22. The crystal structures solved previously for variant forms of *S. cerevisiae* IA₃ complexed with saccharopepsin (3, 4) revealed that the lysine at position 18 in the wild-type *S. cerevisiae* IA₃ sequence is unable to position its charged ε-amino group optimally to satisfy its three terminal hydrogen bond donors and/or to neutralize its positive charge by interacting exclusively with enzyme residues (Fig. 3C). It is thus dependent on D22 of IA₃ and water 1 as hydrogen bond partners in addition to Asp-32 of the enzyme (Fig. 3C). Water 1 is in contact with the catalytic water 2 (in Fig. 3C), which is held in place by the carboxyls of the two catalytic Asp residues (Asp-32 and Asp-215) in all aspartic proteinases (1, 4, 11). The carboxyl group of D22 also contacts the side chain of Tyr-75 and water molecules 3 and 4 (in Fig. 3C). Water 4 serves as a connecting link between the firmly positioned catalytic water 2 and a further water 5 that in turn is in hydrogen bond contact with the side chain OH of Tyr-189 of the proteinase (Fig. 3C). This tight hydrogen bond network between the K18/D22 pair and the enzyme thus anchors IA₃ directly through Asp-32 and Tyr-75 and indirectly through two water-mediated contacts with the catalytic water 2 (Fig. 3C). Any translational or rotational movement of the inhibitor within the active site would be resisted by these tightly established hydrogen bonds and would be costly in terms of binding energy. The inhibitor is thus essentially deadlocked in its position relative to the proteinase.

In contrast, when the lysine is at residue 22, as in the M18/K22 mutant IA₃ from *S. cerevisiae*, the ε-NH₃⁺ group can reach directly between the carboxyl groups of the two catalytic residues (Asp-32 and Asp-215) of saccharopepsin, displacing the catalytic water molecule and picking up three hydrogen bonds to neutralize its positive charge and satisfy its three hydrogen bond donors, as described previously (6). In this case, since there is only one inhibitor residue involved in forming hydrogen bonds with the two catalytic Asp residues that are, by def-
inition, present in the active site of every aspartic proteinase, subtle translation or rotation of the inhibitor might be permitted without a large interaction energy decrease, resulting in the relaxed IA₃ specificity observed in sequences with a lysine at residue 22 and a hydrophobic residue at position 18. This specificity-lowering effect is facilitated by the co-introduction of a hydrophobic residue at position 18, which contributes its binding energy via nondirected hydrophobic interactions, allowing limited inhibitor motion without loss of binding energy. Thus, the M18/K22 combination can give rise to a relaxation of the stringency imposed by the K18/D22 pair on the interaction between *S. cerevisiae* IA₃ and its cognate target proteinase. This interpretation is further substantiated by the IA₃ mutants, which contain two hydrophobic residues at positions 18 and 22. They offset increased potency due to optimized location of the inhibitor inside the active site for significantly lowered specificity.

In conclusion then, the key K18/D22 feature of the wild-type *S. cerevisiae* IA₃ that deadlocks the inhibitor in position is interconnected through the extensive hydrogen bond network depicted in Fig. 3C not only to the catalytic Asp-32 and Tyr-75 residues of the enzyme but also to Tyr-189. The precise positioning of this side chain is balanced by the contact made on its other side through the Ala-213 residue that proved to be the key component contributed by the enzyme (Fig. 3C). Through this extended series of contacts, information is thus exchanged between the two protein partners to facilitate their specific interaction and cement the inhibition of the proteinase through the unusual helix-forming mechanism.

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