Turkey ovomucoid third domain with Leu18 in its reactive site is a potent inhibitor of many serine proteinases: subtilisins, chymotrypsins, and elastases. Previous studies showed that an L18K mutation made it a moderately strong inhibitor of trypsin, while an L18E mutation made it a strong inhibitor of Glu-specific Streptomyces griseus proteinase (GluSGP). For human furin substrates the consensus optimal sequence is RXKR1. Therefore the A15R, T17K, and L18R mutations were made in turkey ovomucoid third domain. The mutant inhibits human furin with a $K_m$ of $1.1 \times 10^7$ M$^{-1}$. As human furin catalyzes an obligatory step in human immunodeficiency virus proliferation, this inhibitor, along with the others already available, deserves further study.

Furin is a member of the recently characterized class of subtilisin-related proprotein convertases or SPCs (for a review see Steinert et al. (1992); see also Chan et al. (1982) for nomenclature). Of the described mammalian convertases furin is probably most broadly distributed among various tissues. Like yeast Kex2 (Fuller et al., 1988) it has a COOH-terminal transmembrane domain and is an integral membrane protein. Thus a COOH-terminally truncated fragment has been developed and used for detailed studies on furin in vitro (Molloy et al., 1992). A broad consensus has been reached that the sequence RXKR1 is the optimal sequence for furin (the 1 indicates the scissile bond) (Hosaka et al., 1991; Bresnahan et al., 1993). However, a detailed analysis (Molloy et al., 1992) suggests that the RXKR1 sequence for furin into the reactive site region of turkey ovomucoid third domain by making the A15R, T17K, and L18R replacements (see Fig. 1) might produce a furin inhibitor. At that time an expression system for ovomucoid third domain variants became available at Rutgers. When the mutant was expressed it was found to be a modest inhibitor of the truncated form of human furin (Molloy et al., 1992).

It may be worth pointing out that many other inhibitor frames such as, for example, bovine trypsin inhibitor (Kunitz) or soybean trypsin inhibitor (Kunitz) might have also been chosen as a frame for introduction of the furin consensus sequence into the reactive site region. The reason for choosing OMKTY3 was that it is a powerful inhibitor of subtilisin (Empie and Laskowski, 1982) while the other two inhibitors do not interact with subtilisin well because of the overall structure of their frames (Hirono et al., 1979). As SPCs and therefore furin are subtilisin homologs it may be that OMKTY3 is better suited as a furin inhibitor.

**MATERIALS AND METHODS**

**Expression of Furin—Truncated human furin, terminating at Leu173 and thus omitting the transmembrane domain, was expressed in African Green monkey kidney cells BSC-40 from a vaccinia virus recombinant VV:hFUR713 as described by Molloy et al. (1992). The final purification was on a Pharmacia LKB Biotechnology Inc. MonoQ S 5/5 anion exchange column. The eluted 250-ml fractions were monitored by a furin enzymatic assay using a fluorescent peptide substrate and SDS-PAGE acrylamide gel electrophoresis. The three fractions constituting the peak of activity served as furin source in this research.**
RESULTS AND DISCUSSION

Addition of massive amounts of OMTKY3 (Fig. 1, top left) produced insignificant inhibition of furin activity. We estimate that \( K_a \) for this inhibitor is 200 \( \text{mM} \) or less. Addition of the L18R variant (Fig. 1, middle right) in very large amounts caused small but noticeable inhibition. \( K_a \) is approximately 450 \( \text{mM} \). However, when the A15R,T17K,L18R variant (Fig. 1, bottom left) was employed clear inhibition was observed (Fig. 2). Fitting of these data caused some problems. Normally the operational molarity of our enzymes is measured prior to the experiment either by burst titration or by titration with a very strong inhibitor. Neither is available for human furin. Therefore, an iterative fitting procedure was employed to yield the molar furin concentrations given on the vertical axis of Fig. 2. The equilibrium constant \( K_a \) is 1.1 \( \times 10^3 \text{ M}^{-1} \). As can be seen the fit in Fig. 2 is quite good. A15R,T17K,L18R OMTKY3 is a moderate inhibitor of furin. We believe that in subsequent design iterations it can be improved as more and more is being published about the detailed specificity of furin. Attempts to improve the inhibitor might prove helpful for unraveling this specificity as well, because \( K_a \) values are far more quantitative than most observations on cleavage. However, even with a \( K_a \) of 1.1 \( \times 10^3 \text{ M}^{-1} \) the inhibitor may be useful in various biological studies on the role of furin and on the HIV virus replication. Indeed tests on this inhibitor have begun. It may be worth noting that the fusion protein that was designed to facilitate the expression of OMTKY3 may in fact help to target the inhibitory variant to appropriate cells.

One of the uses of the new "standard mechanism" furin inhibitor is that operational normality of furin can now be determined, albeit in a cumbersome way. Should it turn out to be possible to improve the \( K_a \) to \( >5 \times 10^4 \text{ M}^{-1} \) the operational normality determination will become quite straightforward by simple titration.

The relative ease with which OMTKY3 was converted to a moderate inhibitor of furin raises the question: has nature already developed many such inhibitors? This question becomes more relevant when we recall that the combining regions of most proteinase inhibitors are hypervariable (Laskowski and Kato, 1980; Hill and Hastie, 1987; Laskowski et al., 1987, 1988; Creighton and Darby, 1989). We have therefore searched sequence data bases of standard mechanism protein-proteinase inhibitors for the RXXRJ sequence, where the arrow defines the position of the inhibitor's reactive site. Surprisingly, we found very few, not because R is rare at \( P_1 \) (there are many trypsin inhibitors) but because R is rare at \( P_4 \). In the

FIG. 1. Amino acid sequences of turkey ovomucoid third domain (upper left), of the L18R variant (middle right), and of the A15R,T17K,L18R variant (lower left). These sequences are of the third domains proper (Laskowski et al., 1987), not of the connecting peptide extended third domains. As the extended third domain has been used in crystallographic studies it has become the basis for the numbering system. Thus NH2-terminal Val shown here is residue 6. The "missing" NH2 terminus has no effect on interaction with enzymes (Wieczorek et al., 1987). The \( P_1, P'_1 \) system of Schechter and Berger (1987) is also shown. The arrow indicates the reactive site peptide bond. The 12 residues forming the consensus enzyme-inhibitor contact set (Apostol et al., 1993) are in boldface circles. The mutations introduced in this work are in black.

Expression of OMTKY3 and Its Variants—OMTKY3 and its site-specific mutagenesis variants were expressed as fusion proteins with a protein A fragment in Escherichia coli. The expression system developed at Rutgers will be the subject of another paper and was presented as a poster (Lu et al., 1992). The fusion protein was secreted into the periplasmic space where the disulfide bridges in the OMTKY3 domain form spontaneously. After osmotic shock it was isolated by affinity chromatography on a Pharmacia IgG column. The inhibitory domain was split off by CNBr cleavage in the linker (the only Met in the fusion protein) and isolated by size exclusion and ion exchange chromatography. The three proteins: 6–56 OMTKY3, Arg18 6–56 OMTKY3, and Arg18, Lys17, Arg18 6–56 OMTKY3 (see Fig. 1) were characterized by amino acid analysis, sequencing (Porton 2020) from residue 6 through 19 and by mass spectrometry (Westec) with electrospray injection. All of the analyses, sequences, and molecular masses were in accord with expectations based on Fig. 1.

Concentration and Activity Determinations—Molar concentration of the inhibitor variants was determined by injecting aliquots of the inhibitor solution onto a TSK G2000 analytical size exclusion column. The area under an equal size aliquot of standardized OMTKY3. As OMTKY3 is a powerful inhibitor of many serine proteinases it can be readily standardized.

The activity of human furin was determined by monitoring the hydrolysis of 1.2 \( \times 10^{-4} \text{ M} \) (final concentration) of \( \alpha \)-butoxyloxybenzoylarginylvalylarginylarginyl-4-methylcoumarin-7-amide. The fluorescence emission was measured at 460 nm (10-nm slit width) on a Perkin-Elmer LS55 spectrofluorometer using 370 nm wavelength (10-nm slit width) for excitation. The reactions were conducted in 0.1 x HEPEs, 0.5% Triton X-100, 0.001 x CaCl2, 0.001 x \( \beta \)-mercaptoethanol, pH 7.50, at 30 °C. The enzyme and inhibitor were incubated together in the reaction medium for 2 h to reach equilibrium. Then substrate was added, and substrate hydrolysis was allowed to proceed for 16 h and then stopped by addition of a 90-fold excess of 1 M x ZnCl2 solution. The fluorescence of these solutions was then measured.

The relative ease with which OMTKY3 was converted to a moderate inhibitor of furin raises the question: has nature already developed many such inhibitors? This question becomes more relevant when we recall that the combining regions of most proteinase inhibitors are hypervariable (Laskowski and Kato, 1980; Hill and Hastie, 1987; Laskowski et al., 1987, 1988; Creighton and Darby, 1989). We have therefore searched sequence data bases of standard mechanism protein-proteinase inhibitors for the RXXRJ sequence, where the arrow defines the position of the inhibitor's reactive site. Surprisingly, we found very few, not because R is rare at \( P_1 \) (there are many trypsin inhibitors) but because R is rare at \( P_4 \). In the

FIG. 2. Inhibition of human furin by A15R,T17K,L18R turkey ovomucoid third domain (Fig. 1). The curve drawn through the points is the best fit for \( K_a = 1.1 \times 10^3 \text{ M}^{-1} \), pH 7.5 and temperature is 30 °C (for buffer conditions see "Materials and Methods"). The molar concentrations of the total inhibitor present were measured directly, but the molar concentrations of free furin were obtained from these data by an iterative process. The dashed line is what would be expected if the inhibition were stoichiometric.
large set of over 400 Kazal domain sequences at Purdue, we found only one with P2 R. This sequence also has P1 R and is chicken ovoinhibitor’s fourth domain (Scott et al., 1987). In most inhibitor families we found no RXXR\(^2\) sequences, but they are abundant in the squash inhibitor family. One of these, an inhibitor from watermelon *Citrullus vulgaris* trypsin inhibitor I (Otlewski et al., 1987) was available to us. Therefore we tested *Citrullus vulgaris* trypsin inhibitor I and entire chicken ovoinhibitor for inhibition of furin. Neither was effective. Both have \(\text{RCPR}11\) sequences, and their failure to inhibit may be due to either Pro in the P2 position or to Ile in the P1’ position or to more remote residues. The question of whether evolution avoids producing natural furin inhibitors is still open.

REFERENCES

Apostoli, I., Giletto, A., Komiyama, T., Zhang, W., and Laskowski, M., Jr. (1993) J Protein Chem. 12, in press

Brennahan, P. A., Hayflick, J. S., Molloy, S. S., and Thomas, G. (1993) in Mechanisms of Intracellular Trafficking and Processing of Pro-proteins (Leh, Y. P., ed) pp. 225-250, CRC Press Inc., Boca Raton, FL

Chen S. J., Oliva, A. A., Jr., LaMendola, J., Grena, A., Bode, H., and Steiner, D. F. (1993) Proc. Natl. Acad. Sci. U. S. A. 89, 6678-6682

Creighton, T. E., and Darby, B. (1989) Trends Biochem. Sci. 14, 319-324

Empie, M. W., and Laskowski, Jr., M. (1992) Biochemistry 21, 2274-2284

Fuller, R. S., Sterne, R. E., and Thorner, J. (1986) Annu. Rev. Physiol. 50, 345-362

Hallenberger, S., Bosch, V., Angliker, H., Shaw, E., Klenk, H., and Garten, W. (1992) Nature 360, 358-361

Hill, R. E., and Hastie, N. D. (1987) Nature 326, 96-99

Hirosu, S., Nakamura, K. T., Itaka, Y., and Mitsui, Y. (1979) J. Mol. Biol. 131, 855-869

Hosaka, M., Nagahama, M., Kim, W., Watanabe, T., Hatsuza, K., Ikemizu, J., Murakami, K., and Nakayama, K. (1991) J. Biol. Chem. 266, 12127-12130

Komiya, T., Bigler, T. L., Yoshida, N., Noda, K., and Laskowski, M., Jr. (1991) J. Biol. Chem. 266, 10727-10730

Laskowski, M., Jr., and Kato, I. (1980) Annu. Rev. Biochem. 49, 593-626

Laskowski, M., Jr., Kato, I., Ardelt, W., Cook, J., Denton, A., Empie, M. W., Kohr, W. J., Park, S. J., Parks, K., Schatlaeley, B. L., Schoenberger, O. L., Tashiro, M., Vichot, G., Whatley, H. E., Wieczorek, A., and Wieczorek, M. (1987) Biochemistry 26, 202-221

Laskowski, M., Jr., Kato, I., Kohr, W. J., Park, S. J., Tashiro, M., and Whatley, H. E. (1988) Cold Spring Harbor Symp. Quant. Biol. 53, 545-552

Lo, W., Komiyama, T., Warne, N., Laskowski, Jr., M., Chuang, Y., Rothberg, I., Ryan, K., and Anderson, S. (1992) Abstracts of the 8th Symposium of the Protein Society, Abstr. S191, p. 79, The Protein Society, San Diego

McCune, J. M., Rubin, L. B., Feinberg, M. B., Lieberman, M., Kosek, J. C., Reyes, G. R., and Weissman, I. L. (1988) Cell 53, 55-67

Moehringer, J., Innocencio, N., Rolleto, B., and Moehringer, T. (1993) J. Biol. Chem. 268, 2590-2594

Molloy, S. S., Brennahan, P. A., Leppila, S. I., Klimpke, K. R., and Thomas, G. (1992) J. Biol. Chem. 267, 16386-16402

Otlewski, J., Whittley, H., Polanowski, A., and Wilusz, T. (1987) Biol. Chem. Hoppe-Seyler 368, 1505-1507

Schechter, I., and Berger, A. (1967) Biochem. Biophys. Res. Commun. 27, 157-162

Scott, M. J., Huckabay, C. S., Kato, I., Kohr, W. J., Laskowski, M., Jr., Tsai, M., and O'Malley, B. W. (1987) J. Biol. Chem. 262, 5899-5907

Steiner, D. F., Smeenkens, S. P., Ohagi, S., and Chan, S. J. (1992) J. Biol. Chem. 267, 23435-23438

Stierneke-Greber, A., Vey, M., Angliker, H., Shaw, E., Thomas, G., Roberts, C., Klenk, H., and Garten, W. (1992) EMBO J. 11, 2407-2412

Wieczorek, M., Park, S. J., and Laskowski, M., Jr. (1987) Biochem. Biophys. Res. Commun. 144, 494-504

Yoshida, N., Tsutsumiya, S., Nagata, K., Hirayama, K., Noda, K., and Makisumi, S. (1988) J. Biochem. (Tokyo) 104, 451-456