Protein Chemical and Kinetic Characterization of Recombinant Porcine Ribonuclease Inhibitor Expressed in *Saccharomyces cerevisiae*†

Anna M. Vicentini,‡ Brigitte Kieffer,‡,§ Renate Matthies,‡ Bernd Meyhack,‖ Brian A. Hemmings,‡ Stuart R. Stone,‡ and Jan Hofsteenge*,‡

Friedrich Miescher-Institut, P.O. Box 2543, Basel CH-4002, Switzerland, and Biotechnology Department, Ciba Geigy, Ltd., Basel, Switzerland

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ABSTRACT: A cDNA encoding porcine ribonuclease inhibitor was used to express this protein in yeast under control of the PHO5 promoter. The recombinant protein was purified to homogeneity with a yield of 0.2 mg/g of yeast cells (wet weight) and was found to be indistinguishable from the inhibitor isolated from porcine liver on the basis of the following criteria: the amino acid composition, the number of free sulfhydryl groups, the molecular weight of the native and the denatured protein, peptide mapping, and amino acid sequence analysis of the N- and C-terminal regions of the protein. A simple method was developed for measuring accurately the slow, tight-biding kinetics of the inhibition of ribonuclease by ribonuclease inhibitor. From the dependence of the observed inhibition constant on the substrate concentration, it could be concluded that RI was competitive with the substrate UpA. The dependence of the observed association rate constant on the substrate concentration was consistent with a two-step mechanism in which the substrate only competed in the second (isomerization) step. The values for the inhibition constant for the inhibition of RNase by the recombinant inhibitor, 67 fM, the association rate constant, 1.5×10^8 $M^{-1} \cdot s^{-1}$, and the dissociation rate constant, 8.3×10^{-6} s^{-1}, were in good agreement with those obtained for the porcine liver RNase inhibitor.

Ribonuclease inhibitor (RI)[1] is a ubiquitously distributed protein inhibitor (M_r ~49K) of ribonucleases (RNases) of the pancreatic type [for a review see Blackburn and Moore (1982)]. It also inhibits the activity of angiogenin, a ribonuclease that stimulates blood vessel outgrowth (Shapiro & Vallee, 1987).

RI inhibits RNases by forming a very tight complex with a stoichiometry of 1:1 (Blackburn et al., 1977; Lee et al., 1989a,b). Evidence has been presented that Lys-41 in RNase (Blackburn & Gavilanes, 1980) and the equivalent residue, Lys-40, in angiogenin (Shapiro et al., 1989) are involved in binding RI. Since this lysyl residue plays a crucial role in the catalytic mechanism of RNase, the binding of RI to this residue may, at least partially, cause the observed inhibition. In contrast, the two active-site histidyl residues do not seem to participate in the interaction with RI (Blackburn & Jailkhani, 1979; Shapiro & Vallee, 1989).

Recently, the primary structures of RI from human placenta (Lee et al., 1988; Schneider et al., 1988) and porcine liver (Hofsteenge et al., 1988) have been determined. Both RIs are acidic proteins containing a large number of glutamic acid and leucyl residues. It was found that RI is entirely built from two alternating homologous repeats of 28 and 29 amino acids length. These repeats are characterized primarily by leucyl residues at constant positions, together with other constant more polar residues. This finding showed that RI belongs to the superfamily of proteins containing leucine-rich repeats (Takahashi et al., 1985; Lopez et al., 1987; Titani et al., 1987; Hashimoto et al., 1988; Reinke et al., 1988; Kataoka et al., 1985). The proteins of this superfamily, which have widely divergent functions, range from yeast adenylate cyclase (Kataoka et al., 1988) to chaoptin, an extracellular matrix protein in *Drosophila* (Reinke et al., 1988). However, for most of these proteins it has been established that they can interact with other proteins, and the hypothesis has been put forward that the common (leucyl) residues in the repeats form a constant structural scaffold and that the more polar and varied residues in between form loops that define a particular protein–protein interaction (Hofsteenge et al., 1988; Schneider et al., 1988).

To facilitate the testing of this hypothesis, and to study further structure–function relationships of the RI by means of site-directed mutagenesis, we have isolated a cDNA encoding the porcine RI and expressed it in *Saccharomyces cerevisiae*. The recombinant protein (r-RI) was characterized by protein chemical methods. Moreover, we developed a versatile and accurate method to study the slow, tight-binding kinetics of the inhibition of RNase A by RI and have used it to characterize both the recombinant and natural inhibitor molecules. These studies complement those of Lee and Vallee (1989, 1990) on recombinant human placental RI.

Experimental Procedures

Materials. Bovine pancreatic RNase A was obtained from Boehringer, Mannheim and purified by affinity chromatography (Wierenga et al., 1973) to ensure that the preparation contained only active molecules. The protein concentration was determined from the absorbance at 280 nm. The catalytic constant (k_{cat}) for the hydrolysis of the dinucleotide uridyl-3′,5′-adenosine (UpA) was found to be 2956 ± 22 s^{-1}.

UpA was obtained from Sigma, St. Louis, MO, and was further purified to remove uridine cyclic 2′,3′-phosphate (U>p)

† The nucleic acid sequence in this paper has been submitted to GenBank under Accession Number J02925.

* Author to whom correspondence should be addressed.

‡ Friedrich Miescher-Institut.

§ Present address: Ecole Supérieure de Biotechnologie de Strasbourg, 11 rue Humann, 67000 Strasbourg, France.

‖ Ciba Geigy, Ltd.

[1] Abbreviations: BNPS-skatole, 2-[(2-nitrophenyl)sulfenyl]-3-bromo-3-methylindolenine; DTT, dithiothreitol; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; *I*, ionic strength; L-RI, ribonuclease inhibitor isolated from porcine liver; MES, 2-(*N*-morpholino)-ethanesulfonic acid; RI, ribonuclease inhibitor; RNase, ribonuclease; SDS, sodium dodecyl sulfate; UpA, uridylyl-3′,5′-adenosine; U>p, uridine cyclic 2′,3′-phosphate; r-RI, recombinant RI isolated from yeast.

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1    AAGCTTGAATTCACATATGAACCTGGACATCCATTGCGAGCAGCTGAGCGACGCCCGGTGGACCGAGCTGCTGCCCCTGCTGCAGCAGTACGAGGTGGTG   100
1                  M  N  L  D  I  H  C  E  Q  L  S  D  A  R  W  T  E  L  L  P  L  L  Q  Q  Y  E  V  V     28

101  CGGCTCGACGACTGCGGCCTGACCGAGGAGCATTGCAAGGACATCGGCAGCGCCCTGCGGGCCAACCCCAGCCTGACCGAGCTGTGCCTGCGGACCAACG   200
29   R  L  D  D  C  G  L  T  E  E  H  C  K  D  I  G  S  A  L  R  A  N  P  S  L  T  E  L  C  L  R  T  N  E    62

201  AGCTGGGCGACGCCGGCGTGCATCTGGTGCTGCAGGGCCTGCAGAGCCCCACCTGCAAGATCCAGAAGCTCAGCCTGCAGAACTGCTCCCTGACCGAGGC   300
63     L  G  D  A  G  V  H  L  V  L  Q  G  L  Q  S  P  T  C  K  I  Q  K  L  S  L  Q  N  C  S  L  T  E  A    95

301  GGGCTGCGGGGTCCTGCCCAGCACGCTGCGCTCCCTGCCCACGCTGCGGGAGCTGCATCTCAGCGACAACCCACTGGGGGACGCCGGCCTGCGGCTGCTC   400
96   G  C  G  V  L  P  S  T  L  R  S  L  P  T  L  R  E  L  H  L  S  D  N  P  L  G  D  A  G  L  R  L  L    128

401  TGTGAGGGGCTCCTGGACCCCCAGTGCCACCTGGAGAAGCTGCAGTTGGAGTACTGCCGCCTGACGGCCGCCAGCTGCGAGCCCCTGGCCTCGGTGCTCA   500
129  C  E  G  L  L  D  P  Q  C  H  L  E  K  L  Q  L  E  Y  C  R  L  T  A  A  S  C  E  P  L  A  S  V  L  R   163

501  GGGCCACGCGGGCCTTGAAGGAGCTCACGGTGAGCAACAACGACATCGGCGAGGCCGGCGCCCGGGTGCTGGGCCAGGGTCTGGCAGACTCTGCCTGCCA   600
163    A  T  R  A  L  K  E  L  T  V  S  N  N  D  I  G  E  A  G  A  R  V  L  G  Q  G  L  A  D  S  A  C  Q   195

601  GCTGGAGACGCTCAGGCTGGAGAACTGCGGTCTCACGCCAGCCAACTGCAAAGACCTGTGCGGAATTGTGGCCTCCCAGGCCTCGCTGAGGGAGCTTGAC   700
196  L  E  T  L  R  L  E  N  C  G  L  T  P  A  N  C  K  D  L  C  G  I  V  A  S  Q  A  S  L  R  E  L  D    228

701  CTGGGCAGCAACGGGCTGGGCGACGCGGGCATAGCCGAGCTGTGCCCCGGGCTCTTGAGCCCCGCCTCCCGCCTCAAGACCCTGTGGCTCTGGGAGTGTG   800
229  L  G  S  N  G  L  G  D  A  G  I  A  E  L  C  P  G  L  L  S  P  A  S  R  L  K  T  L  W  L  W  E  C  D   262

801  ACATCACCGCCAGTGGCTGCAGAGACCTCTGCCGTGTCCTCCAGGCCAAGGAGACCCTGAAGGAGCTCAGTCTGGCGGGCAACAAGCTGGGCGACGAGGG   900
263    I  T  A  S  G  C  R  D  L  C  R  V  L  Q  A  K  E  T  L  K  E  L  S  L  A  G  N  K  L  G  D  E  G   295

901  CGCCCGGCTGCTGTGCGAGAGCCTGCTGCAGCCCGGCTGCCAGCTGGAGTCCCTGTGGGTGAAGTCCTGCAGCCTCACGGCGGCCTGCTGCCAGCACGTC  1000
296  A  R  L  L  C  E  S  L  L  Q  P  G  C  Q  L  E  S  L  W  V  K  S  C  S  L  T  A  A  C  C  Q  H  V    328

1001 AGCTTGATGCTGACCCAGAACAAGCATCTCCTGGAACTTCAGTTGAGCAGCAACAAGCTGGGTGACTCTGGCATCCAGGAGCTGTGCCAGGCCCTGAGCC  1100
329  S  L  M  L  T  Q  N  K  H  L  L  E  L  Q  L  S  S  N  K  L  G  D  S  G  I  Q  E  L  C  Q  A  L  S  Q   362

1101 AGCCGGGCACCACACTGCGGGTGCTCTGTCTTGGGGACTGTGAGGTGACCAACAGCGGCTGCAGCAGCCTCGCCTCGCTCCTGCTGGCCAACCGCAGCCT  1200
363    P  G  T  T  L  R  V  L  C  L  G  D  C  E  V  T  N  S  G  C  S  S  L  A  S  L  L  L  A  N  R  S  L   396

1201 GCGAGAGCTGGACCTGAGCAACAACTGTGTGGGCGACCCGGGCGTCCTGCAGCTGCTGGGGAGCCTGGAGCAGCCGGGCTGCGCCCTGGAGCAGCTGGTC  1300
396  R  E  L  D  L  S  N  N  C  V  G  D  P  G  V  L  Q  L  L  G  S  L  E  Q  P  G  C  A  L  E  Q  L  V    428

1301 CTGTACGACACCTACTGGACGGAGGAGGTGGAGGACCGCCTGCAGGCCCTGGAGGGGAGCAAGCCCGGCCTGAGGGTCATCTCCTGAGAGGCTCCTTTTC  1400
429  L  Y  D  T  Y  W  T  E  E  V  E  D  R  L  Q  A  L  E  G  S  K  P  G  L  R  V  I  S                    456

1401 CCTGGACGGCCTTCCTCCCCCACGGGGATCCCAGCTGTACTCGAGAAATGCTCACATCACCTTACTCCCGTGAGAGAACTTTCGGCACTCTACTTAGTAA  1500

1501 AACACCTTTTGGGCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAGGAATTC 1574
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FIGURE 1: Nucleotide sequence of the cDNA coding for the porcine RNase inhibitor. A cDNA coding for residues 71–456 of the porcine RNase inhibitor was isolated from a library prepared from RNA from porcine kidney cells (LLC-PK_1). The deduced amino acid sequence is shown under the nucleotide sequence. The sequence from nucleotides 17–258 was synthesized on the basis of the amino acid sequence of residues 1–81 of the procine liver RNase inhibitor (Hofsteenge et al., 1988). This sequence and that of the linker region (1–16) are shown in italics.

whose content could amount to 2.5% of that of UpA. UpA (10 mg) was applied to a Whatman ODS-3 C_{18} column (1 × 25 cm) equilibrated in 100 mM NH_4HCO_3, pH 7.0. The dinucleotide was eluted with a linear gradient of CH_3CN (0–20% over 30 min) at a flow rate of 3 mL/min. By use of this procedure, UpA virtually free of U>p (<0.05%) and suitable for kinetic experiments was obtained. NH_4HCO_3 was removed by repeated lyophilization from sterile water that had been treated with diethyl pyrocarbonate.

Construction of the Yeast Expression Plasmid. A cDNA library prepared from poly(A)+ RNA from porcine kidney cells (LLC-PK_1; Hemmings et al., 1986) was screened with a 108 bp probe that corresponded to the amino acid sequence of residues 163–198 of porcine liver RI (L-RI; Hofsteenge et al., 1988) according to previously described methods (Stone et al., 1987b). The clone λpRI3 containing the longest open reading frame (1340 bp) was sequenced on both strands by the dideoxynucleotide chain termination method (Sanger et al., 1977) using bacteriophage T7 DNA polymerase and [α-^{35}S]thio-dATP. It was found to encode residues 71-456 of the porcine RI (Figure 1). A full-length cDNA was constructed by ligating a synthetic oligonucleotide coding for amino acid residues 1–81 of L-RI to the 1127 bp *Xho*II/*Eco*RI fragment isolated from λpRI3 which codes for residues 82–456. An *Xho*I restriction site was created downstream of the translation termination codon by site-directed mutagenesis. The *Eco*-

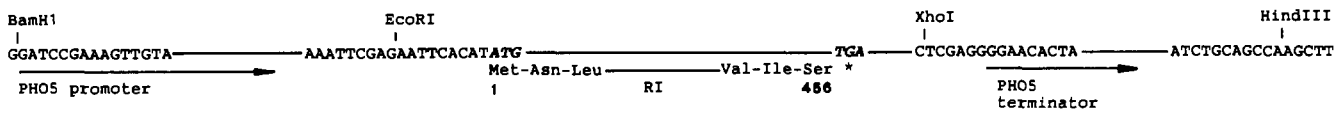

FIGURE 2: Structure of the expression cassette for RNase inhibitor. The cDNA coding for the porcine RI was ligated into the yeast–*E. coli* shuttle vector pJDB207/PHO5-RIT12 between the *Eco*RI and *Xho*I site. Details of this vector have been described under Experimental Procedures. The resulting expression plasmid has been denoted pJDB207/PHO5-RI.

RI/*Xho*I fragment was cloned into the yeast vector pJDB207/PHO5-RIT12 (Figure 2). Plasmid pJDB207/PHO5-RIT12 was derived from the multicopy yeast plasmid pJDB207 (Beggs, 1981). Part of the tetracylin resistance gene and some 2μ sequences between the *Bam*HI and *Hin*dIII sites were replaced by a new DNA insert. This insert consisted of the 534 bp promotor of the inducible yeast acid phosphatase *PHO5* (Bajwa et al., 1984) with an *Eco*RI site introduced at position −8, the invertase signal sequence, and a 125 bp *PHO5* transcriptional terminator sequence (nucleotide positions 1364–1488; Bajwa et al., 1984), flanked 5′ by an *Xho*I restriction site and 3′ by a *Hin*dIII site. The cDNA coding for L-RI was inserted into this plasmid between the *Eco*RI and *Xho*I restriction sites, resulting in expression plasmid pJDB207/PHO5-RI (Figure 2). The correct identity of the plasmid was determined by restriction analysis and sequencing of the 5′-region of the cDNA coding for L-RI.

Expression in Yeast and Purification of Recombinant RI. The yeast strain GRF18 (*α his3-11 his3-15 leu2-3 leu2-112 can*^{R}; Hinnen et al., 1978) was transformed with the expression plasmid pJDB207/PHO5-RI, and recombinant colonies were selected for their ability to grow on leucine-deficient regeneration agar. Four individual yeast transformants were precultured at 25 °C in high-phosphate medium (Haguenauer-Tsapis & Hinnen, 1984). Cells were collected, washed, and shifted to low-phosphate medium. The induction of RI synthesis at 5, 24, and 48 h after the transfer was followed by Western analysis of intracellular extracts. Subsequently, cellular suspensions (10^8 cells/mL) in 50 mM Tris-HCl, pH 7.5, containing 1 mM DTT and 4 mM Zwittergent 3-14 (Navarrette & Serrano, 1984) were lysed with 1 g/mL 0.5–0.75 mm diameter glass beads in a KDL Dyno mill shaker. The cellular debris was removed by centrifugation at 3000*g* for 6 min at 4 °C. The extract was diluted 3-fold with 50 mM potassium phosphate, pH 6.4, containing 0.75 M NaCl, 5 mM DTT, and 1 mM EDTA and purified on an RNase–Sepharose column as described by Burton and Fucci (1982). The protein was further purified as described previously (Hofsteenge et al., 1988).

Molecular Weight Determinations. The molecular weight of the native recombinant RI (r-RI) was determined by using gel filtration on a Superose 12 column (Pharmacia, Uppsala, Sweden) equilibrated in 10 mM sodium phosphate, pH 7.4, containing 200 mM NaCl. The column was calibrated with a set of molecular weight standards: IgG, bovine serum albumin, ovalbumin, bovine pancreatic trypsin inhibitor, and myoglobin. The molecular weight of the denatured and reduced protein was determined by SDS–PAGE (Laemmli, 1970). Gels were stained with Coomassie brilliant blue and scanned at 600 nm by using a Shimadzu CS 930 TLC scanner.

Protein Chemical Characterization. The two large CNBr fragments of r-RI, as well as a subfragment of CB3, were generated and purified as described previously (Hofsteenge et al., 1988). Methods for the determination of amino acid sequences and compositions have been described previously (Hofsteenge et al., 1988).

Titration of Sulfhydryl Groups. DTT was removed from the protein solution under nonoxidizing conditions by centrifiguation of 100-μL aliquots through a Sephadex G-10 column (1 mL) equilibrated in 50 mM NH_4HCO_3 in a capped tube that had been thoroughly flushed with argon. Subsequently, the concentration of protein-associated thiol groups was determined in 100 mM HEPES buffer, pH 7.25, containing 1 mM EDTA and 0.6 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB; Ellman, 1959; Ridles et al., 1983) in the absence or presence of 6 M guanidinium chloride. The protein concentration was determined by amino acid analysis.

RNase and RI Assay. The amount of r-RI in crude extracts was determined by adding aliquots of a suitable dilution to 0.5 pmol of RNase in a total volume of 100 μL of buffer [50 mM Tris-HCl, pH 7.5, containing 1 mM EDTA, 1 mM DTT, 100 mM NaCl, 0.1% poly(ethylene glycol) (M_r 6000), and 0.2 mg/mL bovine serum albumin]. After incubation for 30 min at room temperature the residual RNase activity was determined by the method of Shapira (1962).

For kinetic studies using purified L-RI or r-RI, the following RNase assay was used. The activity of 10 pM RNase was determined by measuring the hydrolysis of uridylyl-3′,5′-adenosine (UpA) as a function of time in 50 mM MES–NaOH, pH 6.0, containing 125 mM NaCl (I = 0.150), 1 mM EDTA, 0.2 mM DTT, 0.1% poly(ethylene glycol) (M_r 6000), 0.2 mg of bovine serum albumin/mL. The reactions were performed at 25 °C in sterile Eppendorf tubes in a thermostated heating block. The reactions were always started by the addition of RNase. Under the conditions described the enzyme was stable for at least 3 h as determined by the progress curve method of Selwyn (1965). To stop the reaction, 50-μL aliquots were mixed with an equal volume of 4 M guanidinium thiocyanate that had been treated three times with charcoal. To determine the concentration of products [cyclic 2′,3′-UMP (U>p) or adenosine], the reaction mixture was fractionated by reversed-phase HPLC on a Whatman ODS-3 C_{18} column using a Hewlett Packard Model 1090L chromatograph equipped with an automatic sample injector. The column was kept at 40 °C and was equilibrated in 150 mM potassium phosphate, pH 4.7 (buffer A). The flow rate was 0.8 mL/min. Under these conditions, cyclic 2′,3′-UMP (U>p) eluted isocratically at approximately 8 min, completely separated from interfering peaks. A steep gradient of acetonitrile (0–20% over 1 min) in buffer A was used to elute UpA and adenosine and to regenerate the column. U>p was detected at 260 nm, and peak areas were determined by integration and converted into concentrations by using a standard plot that was constructed from known amounts of U>p versus peak area. This plot was linear over the range used in these studies and had a correlation coefficient of 1.000. Consumption of substrate never exceeded 4% in the experiments reported in this study.

Data Analysis

Tight-Binding Inhibition of RNase by RI. The inhibition of RNase by RI in the presence of the substrate (UpA) can be depicted by Scheme I, where K_m is the Michaelis constant for the substrate, k_{cat} the catalytic constant, and K_i the dissociation constant of the RNase–RI complex. The value of the observed K_i (K_i') was determined by incubating a constant

Scheme I

$$EI \underset{k_3}{\overset{k_4}{\rightleftharpoons}} I + E + S \overset{K_m}{\rightleftharpoons} ES \xrightarrow{k_{cat}} E + P$$

$$K_i = k_4/k_3$$

Scheme II

$$EI^* \underset{k_5}{\overset{k_6}{\rightleftharpoons}} EI \underset{k_3}{\overset{k_4}{\rightleftharpoons}} I + E + S \overset{K_m}{\rightleftharpoons} ES \xrightarrow{k_{cat}} E + P$$

$$K_i = k_4/k_3$$

$$K_i^* = K_i \frac{k_6}{k_5 + k_6}$$

amount of RNase (10 pM) with increasing amounts of RI in the presence of 1.7 mM UpA. Ten to twelve inhibitor concentrations were used, four of which were below the enzyme concentration and the remainder above. The steady-state rate of substrate hydrolysis (v_s) was determined from the change in concentration of U>p between 60 and 120 min of reaction. Since RI inhibits RNase at concentrations comparable to that of the enzyme, it is termed a tight-binding inhibitor (Morrison & Walsh, 1988). For such an inhibitor the value of v_s as a function of the inhibitor concentration is given by eq 1

$$v_s = \frac{v_0}{2E_t}[[(K_i' + xI_x - E_t)^2 + 4K_i'E_t]^{1/2} - (K_i' + xI_x - E_t)] \quad (1)$$

(Morrison, 1969; Henderson, 1972; Williams & Morrison, 1979), where v_0 is the velocity observed in the absence of inhibitor, K_i' is the observed dissociation constant, E_t is the total RNase concentration, I_x is the inhibitor concentration in terms of weight per volume, and x is a factor such that the product xI_x is the molar concentration of active RI. The measured values of v_s were fitted to eq 1 by nonlinear regression as described previously (Stone & Hofsteenge, 1986). This yielded a value for K_i' and the molar concentration of active RI molecules.

Slow, Tight-Binding Inhibition. Two basic mechanisms for slow binding inhibition have been recognized [Cha, 1976; for a recent review see Morrison and Walsh (1988)]. In mechanism A, the inhibitor and the enzyme associate as depicted in Scheme I. In mechanism B, an initial complex is formed between the enzyme and the inhibitor (EI in Scheme II), and this complex subsequently isomerizes to form the tight complex EI*.

If the inhibitor is competitive with respect to the substrate, the two mechanisms may be distinguished by the effect of the inhibitor on the initial velocity of product formation (Morrison & Walsh, 1988). Whereas this velocity is independent of the inhibitor concentration for mechanism A, it varies hyperbolically in mechanism B. It has been pointed out previously that mechanism B cannot be distinguished from A if K_i is very much greater than the observed K_i^* and the inhibitor concentration is varied in the range of observed K_i^*. In that case, the data can be well described by the equation for mechanism A (Morrison & Stone, 1985).

The amount of U>p formed at 19 time points up to 120 min was determined at 6 different inhibitor concentrations. No variation of the initial velocity of U>p formation with the inhibitor concentration was observed. Therefore, the data were fitted to eq 2 by nonlinear regression as described previously

$$P = v_s t + \frac{(v_0 - v_s)(1 - d)}{dk'} \log \frac{1 - d\exp(-k't)}{1 - d} \quad (2)$$

(Stone & Hofsteenge, 1986). This equation (Cha, 1976; Williams et al., 1979) describes slow, tight-binding inhibition according to mechanism A (Scheme I), where P is the amount of product at time t, d is a function of E_t, I_t, and the observed inhibition constant (K_i'), and k' is a function of these parameters and the observed second-order association rate constant (k_{obs}). This analysis yielded estimates for the values of K_i' and k_{obs}. To obtain the true values of these constants, they have to be corrected for the presence of substrate. This correction is dependent on the mechanism of inhibition and has been described under Results and Discussion.

K_m and k_{cat} values for the substrate UpA were determined from initial velocity data by using previously published methods (Hofsteenge et al., 1986).

Results and Discussion

cDNA Cloning and Expression. A cDNA library constructed from poly(A)+ RNA prepared from porcine kidney LLC-PK_1 cells was screened with the 108 bp probe described above. The choice of this library was based on the finding that the activity of RI per mg of protein in crude extracts of LLC-PK_1 cells was approximately 10 times higher than that found in tissues such as porcine liver. Five positive clones were isolated. The longest cDNA clone (λpRI3, 1340 bp) was found to code for residues 71–456 of porcine RI (Figure 1). It also contained 125 bp of 3′ noncoding sequence including a poly(A) tail. The deduced amino acid sequence of residues 71–456, shown in Figure 1, was identical with that determined for the protein purified from porcine liver (Hofsteenge et al., 1988). Northern analysis (data not shown) indicated that, in all porcine tissues examined, RI is coded for by a single mRNA with a length of 1.9 kb.

Comparison of the nucleotide sequence of λpRI3 with the cDNA coding for the human placental RI (Lee et al., 1988; Schneider et al., 1988) showed that the two sequences were 86% identical, which can be compared with the 77% identity at the protein level. Interestingly, the cDNAs from both organisms have a very high GC content (65%), which is due to a selective codon usage. For example, 94% of the codons for Leu in the porcine cDNA have G or C in the third position. Moreover, in the porcine cDNA, the codons for Glu, Asp, Gln, and Asn have, with only one exception, C rather than T in the third position. A similar selectivity is observed in the human cDNA (Lee et al., 1988; Schneider et al., 1988).

A cDNA coding for the entire RI molecule was synthesized and cloned into the yeast–*Escherichia coli* shuttle vector pJDB207/PHO5-RIT12 to yield the expression plasmid pJDB207/PHO5-RI (Figure 2; see Experimental Procedures). r-RI was expressed in yeast under the control of the inducible *PHO5* promoter and was expected to accumulate in the cytosol.

Purification of r-RI. The recombinant protein could be purified from yeast extracts according to the procedures described for the proteins from human placenta and mammalian liver (Blackburn, 1979; Burton & Fucci, 1982). The yield of r-RI was approximately 0.2 mg/g of yeast cells (wet weight). The purity of the isolated protein was assessed by SDS–PAGE (Figure 3A). The recombinant protein had the same electrophoretic mobility as porcine L-RI (Figure 3A), and its apparent molecular mass was estimated to be 46 000 Da. Two minor protein bands of lower molecular mass (amounting to approximately 7% of total protein) were observed. These bands were also observed in L-RI and represented degradation products as was shown by western analysis and amino acid sequencing (data not shown).

One of the major problems in producing recombinant proteins is the adventitious or incorrect formation of disulfide

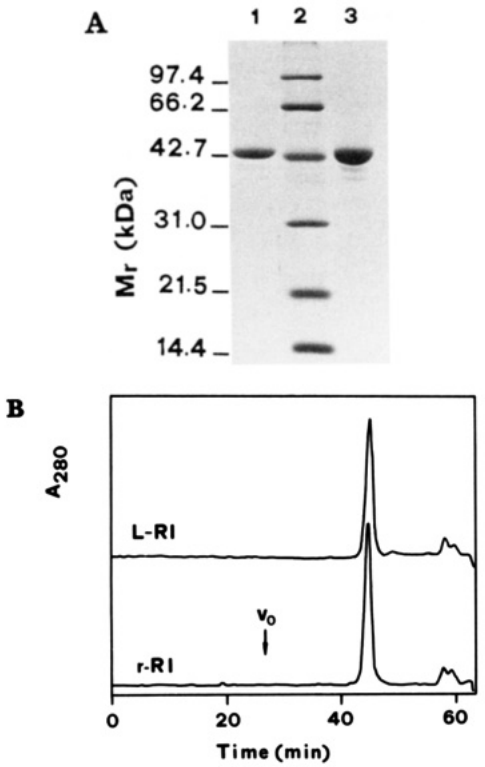

FIGURE 3: Purity and molecular weight of the recombinant RNase inhibitor from yeast. (A) A representitive preparation of purified inhibitor from yeast was compared with that isolated from porcine liver by electrophoresis on a 12.5% SDS–polyacrylamide gel. Lane 1, 1 μg of porcine liver RNase inhibitor; lane 2, standard proteins of the indicated molecular mass; lane 3, 3 μg of recombinant RNase inhibitor. The proteins were visualized by staining with Coomassie brilliant blue. (B) Ten microliters of a solution of recombinant (12.2 μM) or porcine liver (11.0 μM) RNase inhibitor were injected onto a Superose 12 gel filtration column. The column was equilibrated in 10 mM potassium phosphate, pH 7.4, containing 200 mM NaCl and was eluted at a flow rate of 0.3 mL/min.

bridges (Mitraki & King, 1989). Since RI contains a large number of half-cystinyl residues (Lee et al., 1988; see also below) that could oxidize and yield cross-linked species, the molecular weight of native r-RI was determined by gel filtration (Figure 3B). The r-RI eluted at the same position as the L-RI with an apparent molecular mass somewhat smaller (43 kDa) than that calculated from the amino acid sequence (49 kDa). These results showed that the purified recombinant r-RI is a single nonaggregated polypeptide chain.

Protein Chemical Characterization. The protein was further characterized by protein chemical methods to confirm its amino acid sequence and the integrity of its N- and C-terminus. The amino acid composition of the r-RI is shown in Table I and was found to be in agreement with that calculated from the amino acid sequence. The intact protein, both unmodified or reduced and carboxymethylated, was not susceptible to Edman degradation. This suggested that the N-terminal amino acid was blocked at the α-amino group. L-RI has previously been found to be N-terminally blocked with an acetyl group (Hofsteenge et al., 1988). The exact nature of the blocking group in r-RI is not known, but N-terminal acetylation of recombinant proteins in yeast has been found to occur (Hallewell et al., 1987).

CNBr cleavage of r-RI yielded two fragments (rCB2 and rCB3) that eluted at the same position as those obtained from L-RI (Figure 4A). The identity of the fragments from r-RI with those from L-RI was further confirmed by their amino acid composition (Table I). Moreover, the 20 N-terminal amino acid residues of both fragments were found to be

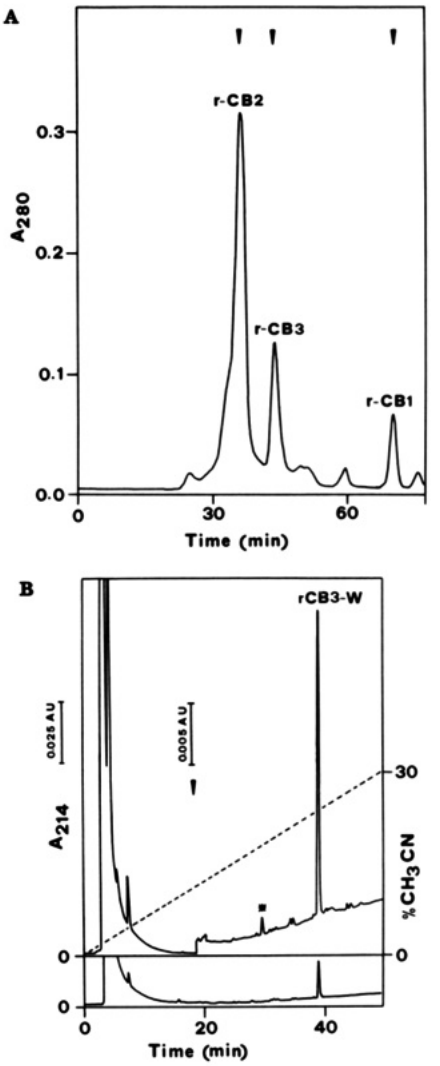

FIGURE 4: Peptide mapping of the recombinant RNase inhibitor. (A) The reduced and carboxymethylated protein was digested with CNBr, and the fragments were fractionated on a Superose 12 column equilibrated in 50 mM NH_4HCO_3 at a flow rate of 0.3 mL/min. The arrows indicate the elution position of the CNBr fragments obtained from the RNase inhibitor from porcine liver. The peak eluting near the included volume contained blocked homoserine. No peptide material was found in this peak by reversed-phase HPLC. (B) The C-terminal fragment, rCB3, was cleaved with BNPS-skatole, and the reaction products were fractionated on a C_4 reversed-phase column (upper panel). The column was equilibrated with 0.1% TFA in water (v/v), and peptides were eluted with a linear gradient of 0–49% CH_3CN in the same solvent, at a flow rate of 1 mL/min. The lower panel shows the chromatogram obtained by cleavage of CB3 isolated from porcine liver RI. The identity of the peptide rCB3-W was confirmed by amino acid analysis (Table I) and Edman degradation. The peak indicated by the asterisk contained the peptide consisting of residues 435–447.

identical with their counterparts from L-RI. Also, the relative amounts of the two fragments, as determined from the ratio of the peak height of CB3 to that of CB2 on the Superose 12 column (Figure 4A), were the same (0.39 and 0.43 for r-RI and L-RI, respectively). Finally, homoserine (rCB1), which resulted from cleavage at the N-terminal methionyl residue, was found upon acid hydrolysis in the included volume of the column.

L-RI has been shown to have a tryptophanyl residues at position 434 (Hofsteenge et al., 1988). Therefore, treatment of the C-terminal CNBr fragment (rCB3) with BNPS-skatole should result in the liberation of a 22 amino acid fragment (rCB3-W). The appearance of smaller fragments would indicate degradation of the C-terminus of r-RI. The result of

Table I: Amino Acid Composition of Recombinant RNase Inhibitor from Yeast and Some of Its Subfragments[a]

amino acid	r-RI	rCB2	rCB3	rCB3-W
Asx	36.2 (40)[b]	26.9 (28)	11.9 (12)	1.1 (1)
Glx	59.3 (60)	41.9 (41)	19.2 (19)	4.9 (5)
CMCys	22.4 (30)	17.3 (24)	4.2 (6)	
Ser	38.3 (38)	22.6 (25)	13.3 (13)	2.1 (2)
Thr	23.1 (23)	16.3 (17)	6.3 (6)	1.2 (1)
Gly	37.9 (37)	24.5 (26)	10.8 (11)	2.1 (2)
Ala	35.9 (32)	29.1 (27)	7.2 (5)	1.4 (1)
Arg	21.7 (22)	15.2 (17)	5.1 (5)	2.0 (2)
Pro	17.6 (16)	12.3 (12)	4.0 (4)	0.9 (1)
Val	20.7 (19)	11.7 (12)	6.5 (7)	1.4 (2)
Met	1.5 (2)	+[c]		
Ile	9.3 (9)	6.2 (7)	1.8 (2)	0.6 (1)
Leu	104.0 (98)	70.8 (70)	29.1 (28)	3.0 (3)
Phe				
Lys	17.6 (14)	10.7 (11)	2.9 (3)	0.9 (1)
His	7.6 (7)	5.1 (6)	1.0 (1)	
Tyr	5.0 (4)	2.1 (2)	1.4 (2)	
Trp	ND (5)	ND (4)	ND (1)	ND

[a] Protein was hydrolyzed for 18 h at 110 °C, and the amino acid composition was determined by the method of Knecht and Chang (1987). [b] The numbers in parentheses indicate the number of residues found in the sequence of the RI from porcine liver (Hofsteenge et al., 1988). [c] Found as homoserine.

Table II: Sulfhydryl Titration of Recombinant and Porcine Liver RNase Inhibitor

form of RNase inhibitor	6 M GuHCl	mol of thiol/mol of protein[a]	n[c]
porcine liver	+	29.8 ± 1.3	3
recombinant	+	31.0 ± 0.7	7
recombinant	-	31.1 ± 0.5[b]	4

[a] The protein and thiol contents were determined as described under Experimental Procedures, and the ratios are given together with their standard deviations. [b] This value was obtained after the reaction had reached a plateau at approximately 40 min. [c] Number of determinations of one representative protein preparation.

cleavage of rCB3 with BNPS-skatole is shown in Figure 4B. The major peptide isolated from rCB3 eluted at the same position as that isolated from CB3 of L-RI. Its amino acid composition (Table I) and amino acid sequence confirmed its identity as residues 435–456. A minor peptide (marked with an asterisk) that was not found in the BNPS-skatole digest of L-RI eluted at 30 min (Figure 4B). This peptide was identified by amino acid analysis as residues 435–447 and was present at about 6% of the amount of rCB3-W. These results show that approximately 94% of the r-RI molecules had the same C-terminus as L-RI, i.e., -Val-Ile-Ser, and that about 6% of the protein was isolated in a form that lacked the nine C-terminal residues. At present, it is unclear whether this deletion has an effect on the activity of the protein.

It has been found for RI isolated from human placenta that all of the 32 half-cystinyl residues occur as free thiol groups (Lee et al., 1988). The 30 half-cystinyl residues in porcine L-RI occurred also as free thiols (29.8 ± 1.3 mol of SH/mol of L-RI; Table II). Titration of r-RI showed the presence of 31.0 ± 0.6 mol of SH/mol of r-RI (Table II). This number of free thiol groups was found both in the presence and in the absence of denaturating agents. However, in the latter case, reaction of L-RI or r-RI with DTNB was completed only after 40 min, and the kinetics were heterogeneous. These observations indicated that not all thiol groups are equally reactive or accessible to the modifying reagent. The fact that all half-cystinyl residues in r-RI occur as free thiol is consistent with the observation that no cross-linked r-RI was found in the preparation (Figure 3B).

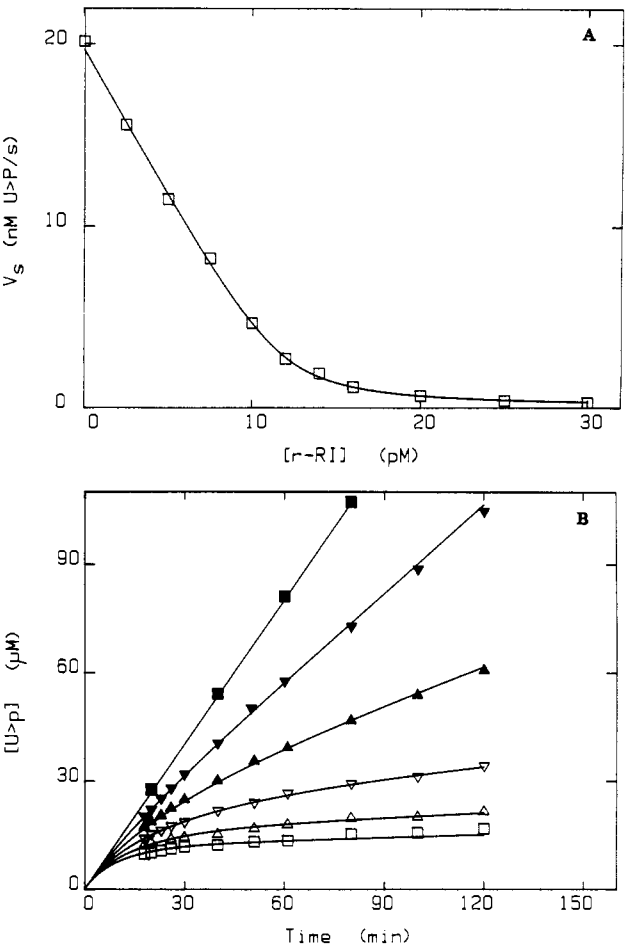

FIGURE 5: (A) Effect of recombinant RNase inhibitor on the steady-state velocity of the hydrolysis of the substrate uridylyl-3′,5′-adenosine. Different concentrations of inhibitor were preincubated with the substrate (UpA; final concentration 1.7 mM), and the reaction was started by the addition of RNase (final concentration 9.2 pM). The steady-state rate of substrate hydrolysis was determined from the concentrations of product at 60 and 120 min. The line shows the best fit of the data to eq 1 (see Data Analysis), with K_i' = 231 fM and v_0 = 2.14 nM·s^{-1}·pM^{-1} RNase. (B) Slow tight-binding inhibition of RNase by recombinant RNase inhibitor. Different concentrations of recombinant RNase inhibitor were preincubated with the substrate (UpA; final concentration 2.3 mM), and the reaction was started by the addition of RNase to a final concentration of 10 pM. At the indicated time points aliquots of the reaction mixture were stopped (see Experimental Procedures) and analyzed for the concentration of product. Time points at 2, 4, 6, and 8 min are not shown for the sake of clarity but were used in the analysis to estimate the kinetic constants. The inhibitor concentrations were as follows: (■) 0 pM; (▼) 4 pM; (▲) 8 pM; (▽) 12 pM; (△) 16 pM; (□) 20 pM. The lines represent the best fit of the data to eq 2, with the parameters given in Table III.

Inhibition of Pancreatic RNase A. The steady-state rate (v_s) of hydrolysis of the substrate UpA by RNase in the presence of increasing amounts of r-RI is shown in Figure 5A. The data were fitted to eq 1 (see Data Analysis) which yielded a value for the observed inhibition constant (K_i') and for the molar concentration of the r-RI in the stock solution. Comparison of the latter with the concentration obtained by amino acid analysis showed that the protein was 94% active in the preparation used (the average value for three different preparations was 92 ± 3%).

Using the experimental approach described under Experimental Procedures, it was also possible to follow the time course of inhibition of RNase by L-RI or r-RI in the presence of UpA. The amount of product formed at 19 time points up to 120 min was determined at 6 different inhibitor concen-

Table III: Kinetic Constants for the Inhibition of RNase by Recombinant and Porcine Liver RNase Inhibitor[a]

form of RNase inhibitor	K_i (fM)	$k_{on} \times 10^{-8}$ ($M^{-1}\cdot s^{-1}$)	$k_{off} \times 10^{6}$ (s^{-1})	method
porcine liver	74 ± 5			1
	59 ± 7	1.66 ± 0.03	9.8 ± 0.2	2
weighted mean	69 ± 4			
recombinant	76 ± 5			1
	55 ± 6	1.5 ± 0.2	8.3 ± 0.2	2
weighted mean	67 ± 4			
human placenta[b]	44	3.4	15	

[a] Assays were performed as described under Experimental Procedures. Method 1 indicates the measurements of the effect of RNase inhibitor on the steady-state rate of hydrolysis of substrate by RNase. Data similar to those presented in Figure 5A were fitted to eq 1 to yield the values for the observed inhibition constants. The true value of this constant (K_i) was obtained by correcting it for the presence of substrate according to eq 3a. Method 2 indicates the measurements of the effect of RNase inhibitor on the progress curve of substrate hydrolysis by RNase. Data similar to those presented in Figure 5B were fitted to eq 2. The values for the observed inhibition and second-order association rate constants were corrected for the presence of substrate according to eqs 3a and 4, respectively, and k_{off} was calculated from the relationship $k_{off} = K_i k_{on}$. The parameters are given together with their standard errors. [b] These values were determined at 25 °C in 100 mM MES, pH 6.0, containing 100 mM NaCl and 1 mM EDTA (I ~0.150) by Lee et al. (1989b).

trations. The data shown in Figure 5B indicated that, under the conditions used here, RI is a slow, tight-binding inhibitor of RNase A. These data fitted well to the equation describing mechanism A (eq 2; see Data Analysis). This analysis yielded an estimate of the values for K_i' and for the observed second-order rate constant (k_{obs}). To obtain the true values of these constants (K_i and k_{on}), the effect of the substrate has to be taken into account.[2] K_i' and k_{obs} were corrected for the presence of substrate according to eq 3a and 4, respectively, and the overall dissociation rate constant (k_{off}) was calculated from the relationship $K_i = k_{off}/k_{on}$. From the tight-binding inhibition data (Figure 5A), a value for K_i of 76 ± 5 fM obtained, whereas the progress curve data (Figure 5B) yielded a value of 59 ± 7 fM. These values were indistinguishable from those found for L-RI, 74 ± 5 fM and 55 ± 6 fM, respectively (Table III). In addition, the values obtained for the association and dissociation rate constants for the r-RI, (1.5 ± 0.2) × 10^8 $M^{-1}\cdot s^{-1}$ and (8.3 ± 0.2) × 10^{-6} s^{-1}, respectively, were the same as those found for the L-RI (Table III). The high value for the association rate constant (10^8–10^9 $M^{-1}\cdot s^{-1}$) suggests that the rate of association is controlled by the rate of diffusion (Nolte et al., 1980).

The values of the dissociation constant of the RNase–RI complex reported in the literature range from 0.3 nM to 44 fM (Blackburn et al., 1977; Burton et al., 1980; Turner et al., 1983; Fominaya et al., 1988; Lee et al., 1989a,b). These differences are partly due to differences in experimental protocols (pH, ionic strength), origin and purity of the enzyme and inhibitor preparations, and type of substrate used (r-RNA, t-RNA, dinucleotides). Moreover, it has been recognized only recently that RI is a tight-binding inhibitor of RNase (Turner et al., 1983, Fominaya et al., 1988; Lee et al., 1989a,b).

We have employed a relatively simple method to obtain a direct estimate of the values for K_i, the association rate constant, and the dissociation rate constant, which are in good agreement with those found by Lee et al. (1989a,b) for human placental RI and RNase A (Table III). The method used by Lee et al. (1989a,b) involved competition for the RI between RNase A and a second RNase-like molecule, angiogenin.

Conclusions

Porcine RI could be expressed in *S. cerevisiae* and purified with a reasonable yield (0.2 mg/g of wet yeast cells). The purified inhibitor (r-RI) was indistinguishable from the one isolated from porcine liver (L-RI) on the basis of a number of protein chemical and kinetic criteria: (i) It consisted mainly of a single, N-terminally blocked, polypeptide chain with the correct N- and C-terminal sequences as shown by amino acid analysis and peptide mapping. (ii) It contained 31 ± 0.4 free thiol groups, compared with 29.8 ± 1.3 in the L-RI. (iii) The values of the kinetic parameters for the inhibition of RNase A by r-RI and L-RI were indistinguishable. The data obtained showed a competitive mode of inhibition of RNase by RI. Furthermore, the results were consistent with a two-step model in which the substrate UpA competes only with the second (isomerization) step.

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Registry No. RI, 39369-21-6; RNase, 9001-99-4.

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[2] The effect of the concentration of UpA on K_i' and k_{obs} was studied at an ionic strength of 0.275 by using L-RI. Data from three or four progress curves at five different substrate concentrations (1.1–6.4 mM) were fitted to eq 2 (see Data Analysis) to obtain values for K_i' and k_{obs}. If the substrate and RI compete for a binding site on the enzyme, K_i' should vary linearly with the substrate concentration according to eq 3a.

$$K_i' = K_i(1 + S/K_m) \quad (3a)$$

This was indeed observed (data not shown). Analysis of the data yielded a value for K_m of 0.9 ± 0.2 mM and for K_i of 1.5 ± 0.4 pM at this ionic strength. The value for K_m was in good agreement with the value determined directly from initial velocity studies (0.87 ± 0.01 mM). The inverse of k_{obs} also showed a linear dependence on the substrate concentration. For mechanism A (Scheme I), eq 3b should describe this dependence. Analysis of the data gave a value for K_m, 10.3 ± 3 mM,

$$1/k_{obs} = (1/k_3)(1 + S/K_m) \quad (3b)$$

that was nearly 10-fold higher than the one obtained above. Thus, although the progress curve data were well described by eq 2 (mechanism A), the mechanism appeared to be more complex. The inhibition of angiogenin by RI has been shown to occur via a two-step mechanism (mechanism B; Scheme II), and it has been assumed that RNase A behaves in a similar way (Lee et al., 1989a,b). However, the initial complex between enzyme and inhibitor need not necessarily involve the active site of the enzyme, and competition between substrate and inhibitor may occur only during the isomerization step. Such a mode of interaction has previously been found in the case of the protease thrombin and its inhibitor hirudin (Stone & Hofsteenge, 1986; Stone et al., 1987a). For such a mechanism k_{obs} is described by eq 4. In this case the observed

$$1/k_{obs} = \frac{k_4 + k_5}{k_3 k_5} + \frac{S}{K_m}\frac{k_4}{k_3 k_5} \quad (4)$$

value of K_m obtained by fitting the data to eq 3b equals $K_m(1 + k_5/k_4)$ and, by use of a value of 0.87 mM for K_m, the value of the ratio k_5/k_4 could be calculated to be 10.8. The value for the overall association rate constant [$k_{on} = k_3 k_5/(k_4 + k_5)$] was (2.5 ± 0.2) × 10^7 $M^{-1}\cdot s^{-1}$ at this ionic strength.

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