Journal of Microbiology and Biotechnology
Vol. 6. No. 4, 250-254, 1996

The Slow and Tight Binding of MR-387A to Aminopeptidase N

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MR-387A [(2*S*,3*R*)-2-hydroxy-3-amino-4-phenylbutanoyl-L-valyl-L-prolyl-(2,4-*trans*)-L-4-hydroxyproline] reversibly inhibits aminopeptidase N (EC 3.4.11.2) in a process that is remarkable for its unusual degree of time dependence. The time required to inactivate the enzyme by 50% ($t_{1/2}$) for establishing steady-state levels of EI* complex was approximately 5 minutes. This indicates that the inhibition is a slow-binding process. In dissociation experiments of EI* complex, enzymic activity was regained slowly in a quadratic equation, indicating that the inhibition of aminopeptidase N by MR-387A is tight-binding and reversible. Thus, the binding of MR-387A by aminopeptidase N is slow and tight, with K_i (for initial collision complex, EI) and K_i^* (for final tightened complex, EI*) of 2.2×10^{-8} M (from Lineweaver-Burk plot) and 4.4×10^{-10} M (from rate constants), respectively. These data indicate that MR-387A and aminopeptidase N are bound approximately 200-fold more tightly in the final EI* complex than in the initial collision EI complex.

Aminopeptidases catalyze the hydrolysis of amino-terminal amino acids from the amino terminus of protein and peptide substrates. Many aminopeptidases are inhibited in a slow binding process (10, 12, 15, 16) by bestatin [(2*S*,3*R*)-2-hydroxy-3-amino-4-phenylbutanoyl-L-leucine] (14) and amastatin [(2*S*,3*R*)-2-hyroxy-3-amino-5-methylhexanoyl-L-valyl-L-valyl-L-aspartic acid] (1), well known inhibitors, require zinc ions for activity, and are oligomeric, and several appear to have common residues used for metal ion binding and substrate recognition (13). Since aminopeptidase N (or aminopeptidase M, EC 3.4.11.2; abbreviated as AP-N) shares these characteristics (7, 9, 13), it can be considered prototypical of the class of bestatin-inhibitable aminopeptidases.

Recently, in the course of screening for AP-N inhibitors, we isolated novel inhibitors MR-387A and B from culture filtrates of *Streptomyces neyagawaensis* SL-387 (4). MR-387A and B showed the structures to be (2*S*,3*R*)-2-hydroxy-3-amino-4-phenylbutanoyl-L-valyl-L-prolyl-(2,4-*trans*)-L-4-hydroxyproline and (2*S*,3*R*)-2-hydroxy-3-amino-4-phenyl-butanoyl-L-valyl-L-prolyl-L-proline, respectively. They inhibited not only AP-N from porcine kidney microsomes but also AP-N of human fibrosarcoma HT1080 and human myelogenous leukemia K562 cell lines. Furthermore, MR-387A (IC_{50}= 0.20 μM) is 100 times as strong as bestatin (IC_{50}=20.1 μM), and is competitive with the substrate L-leucine-*p*-nitroanilide. Inhibition of AP-N by MR-387A derivatives indicated that the number of amino acids in the inhibitor affects the tightness of binding between the inhibitor and AP-N (5), and suggested that an interaction between the α-hydroxy-β-amino acid residues and enzyme is needed to stabilize a tighter complex between AP-N and bestatin or amastatin (10).

To further evaluate the inhibition characteristics of MR-387A against AP-N, kinetic experiments were carried out. The results are reported herein regarding the kinetic parameters for the slow and tight binding of MR-387A to AP-N.

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Key words: aminopeptidase N, MR-387A, kinetic parameter, slow and tight binding

MATERIALS AND METHODS

Materials

L-Leucine-*p*-nitroanilide (abbreviated as L-Leu-pNA) and AP-N (microsomal leucine aminopeptidase from porcine kidney, Sigma L-0632) were purchased from Sigma Chemical Co. MR-387A was purified from culture filtrates of *Streptomyces neyagawaensis* SL-387 by the procedure previously reported (4). All other chemicals were of analytical grade.

Standard Enzyme Assay

Standard AP-N assay was carried out as reported previously (12).

Time Course of Inhibition of AP-N by MR-387A

Two experiments were carried out to determine the time course of the inhibition of AP-N by MR-387A. The first progress curve (Fig. 1) was generated by addition of 1 mU AP-N (1.5×10^{-9} M subunit) to 3.5×10^{-4} M substrate L-Leu-pNA. The data were plotted as *p*-nitroaniline produced *versus* time. The other assay (Fig. 2) was done by incubation of 10 µl AP-N (1.4×10^{-8} M) with MR-387A (3.5×10^{-6} M) in 100 mM Tris·HCl (pH 7.0). Four µl aliquots were withdrawn at the time periods indicated and diluted 1000-fold into 3.5×10^{-4} M L-Leu-pNA in the same buffer. After incubation for 30 minutes, *p*-nitroaniline liberated by hydrolysis of substrate was monitored at 405 nm.

Dissociation of EI* Complex

Dissociation of tightened AP-N : MR-387A (EI*) complex upon dilution was carried out as follows. Inhibitor and enzyme at a concentration of 3.5×10^{-6} M in 100 mM Tris·HCl, pH 7.0, were incubated until 80% of the original activity was inhibited and then diluted 1000-fold in 3.5×10^{-4} M substrate. The hydrolysis of substrate was monitored with incubation time.

Determination of Kinetic Constants under Pre-steady-state Conditions

Pre-steady-state data were used to determine the type of inhibition and to corroborate the kinetic constants. Reactions were started by the addition of 10 µl of AP-N (1 mU final) to 0.2 ml of 0.045, 0.09, 0.18 and 0.35 mM substrate (final concentrations) each containing 0, 20 and 100 nM MR-387A. Progress curves were plotted as changes of O.D. at 405 nm versus time.

RESULTS AND DISCUSSION

Slow Binding of MR-387A to AP-N

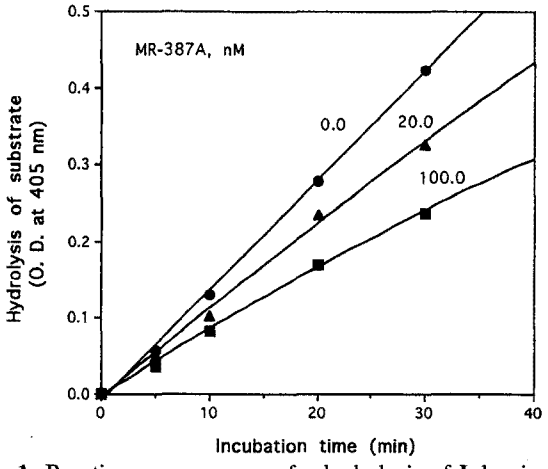

Fig. 1. Reaction process curves for hydrolysis of L-leucine-*p*-nitroanilide by aminopeptidase N in the presence of increasing concentration of MR-387A.

In the absence of inhibitor, the steady-state velocity for hydrolysis of L-Leu-pNA by AP-N was reached immediately (Fig. 1). In the presence of MR-387A, however, there is a slow decrease in reaction rate, which varies as a function of the inhibitor concentration. When the enzyme was incubated with 3.5×10^{-6} M MR-387A and then diluted and assayed, there was a pronounced decrease in enzyme activity resembling irreversible inactivation (Fig. 2).

The slow-binding inhibition can be interpreted by the following scheme described by Rich *et al.* (10).

Where, k_{on} and k_{off} are the rate constants for formation and deformation of EI* complex. The time required to inactivate the enzyme by 50% ($t_{1/2}$) for establishing steady-state levels of EI* complex was approximately 5 minutes under these conditions. Rate constants for bimolecular collisions are dependent on the size (and shape) of both molecules (enzyme and inhibitor) and on diffusion. For molecules of this size, k_1 ranges from 10^7 to 10^8 $M^{-1}s^{-1}$ (8). Given an MR-387A concentration of 2×10^{-8} M and k_1 of 10^7 $M^{-1}s^{-1}$, k_{obsd} for the diffusion-controlled bimolecular collision would be 0.2 s^{-1}, corresponding to a half-life of 3.5 s (=ln $2/k_{obsd}$). This half-life is too short to account for the slow process observed. The $t_{1/2}$ for establishing steady-state levels of EI* complex is much longer than the half-life for the diffusion-controlled bimolecular collision (8). This indicates that the inhibition of AP-N by MR-387A is a slow-binding process exhibiting a mechanism shown in scheme I.

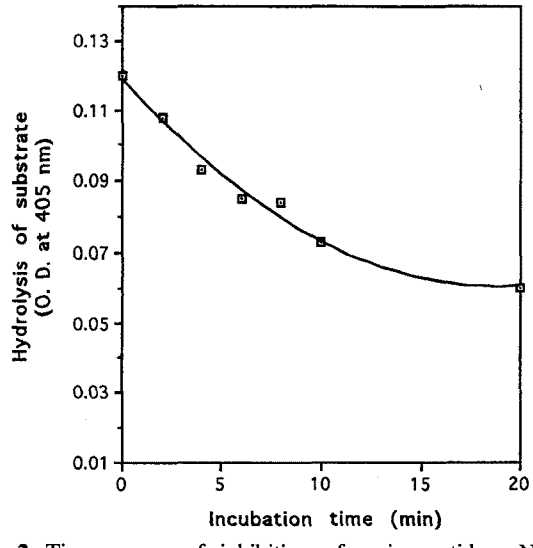

Fig. 2. Time course of inhibition of aminopeptidase N by MR-387A.

The assay was done by incubation of 10 µl AP-N (1.4×10^{-8} M) with MR-387A (3.5×10^{-6} M) in 100 mM Tris·HCl (pH 7.0). 4 µl aliquots were withdrawn at the time periods indicated and diluted 1000-fold into 3.5×10^{-4} M L-Leu-pNA in the same buffer. After incubation for 30 minutes, *p*-nitroaniline liberated by hydrolysis of substrate was monitored at 405 nm.

$$E + S \rightleftharpoons ES \longrightarrow P$$
$$+$$
$$I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EI^*$$
$$k_{on} \quad k_{off}$$

Scheme I.

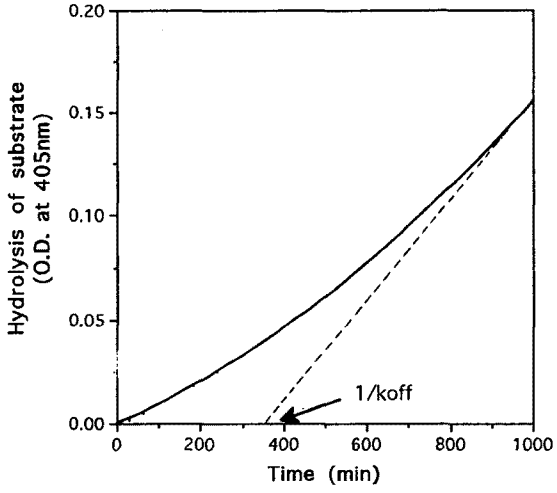

Fig. 3. Dissociation of aminopeptidase N : MR-387A complex upon dilution.

Inhibitor and enzyme at a concentration of 3.5×10^{-6} M in 100 mM Tris·HCl, pH 7.0, were incubated until 80% of the original activity remained and then diluted 1000-fold in 3.5×10^{-4} M substrate. The hydrolysis of substrate was monitored with incubation time. The intercept of an asymptote (·····) on the abcissa is equvalent to the reciprocal of k_{off}.

Tight Binding of MR-387A to AP-N

As shown in scheme I, the slow development of inhibition is caused by slow changes in the initially formed collision complex (EI), leading to a new enzyme-inhibitor complex (EI*) in which the inhibitor is bound more tightly to the enzyme. In order to determine the dissociation constant for dissociation of EI* complex, AP-N (3.5×10^{-6} M) was equilibrated with an excess of MR-387A (3.5×10^{-6} M) for 30 min until 80% of the original activity was inhibited. The complex was then diluted by the addition of a large volume of buffer that contained substrate (3.5×10^{-4} M). The time course for this assay is shown in Fig. 3. Enzymic activity was regained slowly in a quadratic equation (correlation coefficient > 0.99). The slow regain in activity indicates that the inhibition of AP-N by MR-387A is tight-binding and reversible. The intercept of asymptote on the abscissa is equivalent to $1/k_{off}$ as shown by Schloss and Cleland (11). From the data shown in Fig. 3, k_{off} is 3.3×10^{-5} s^{-1}, corresponding to a half-life of 350 min for the dissociation of EI* complex.

Competitive Inhibition of MR-387A to AP-N

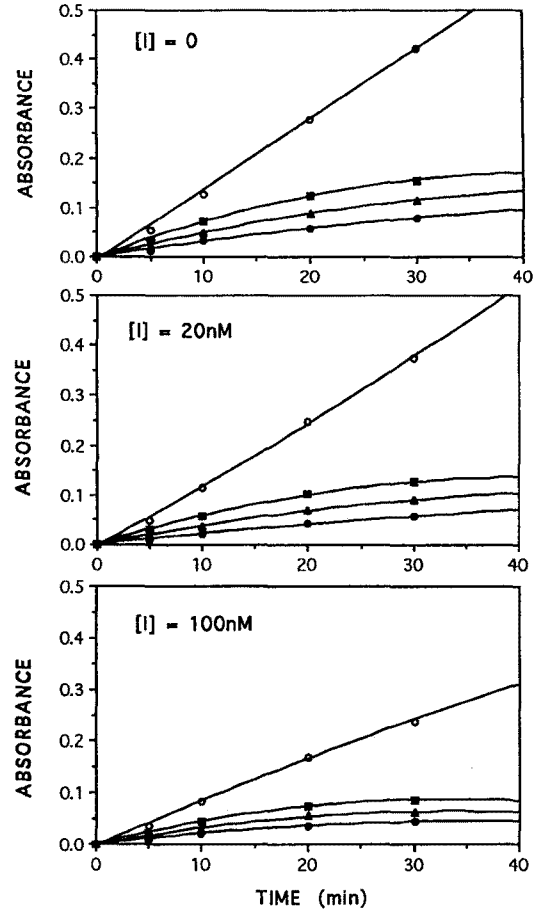

Fig. 4. Reaction process curves for hydrolysis of L-leucine-*p*-nitroanilide (absorbance at 405 nm) by aminopeptidase N in the presence of various concentrations of inhibitor and substrate.

Substrate: L-leucine-*p*-nitroanilide ○, 0.35 mM; ■, 0.18 mM; ▲, 0.09 mM; ●, 0.045 mM. Inhibitor: MR-387A.

A Lineweaver-Burk plot under pre-steady state conditions (Figs. 4 and 5) established that MR-387A is a competitive inhibitor of AP-N with a K_i of 2.2×10^{-8} M.

Cha (3) and Baici (2) derived methods to analyze pre-steady-state kinetic data for slow binding inhibitors and to identify the type of inhibition. A simple scheme which describes slow binding inhibition is shown in scheme I where the dissociation constant for the initial (EI) collision complex is $K_i=k_1/k_2$ and dissociation constant, K_i^*, for the final complex, EI*, is $k_{off}/k_{on}=k_2k_4/k_1k_3$. K_i^* can either be calculated from the ratio of k_{off}/k_{on} as obtained from pre-steady-state experiments or be determined directly using data from both phases of the reaction. The data were fitted with quadratic equations, and velocities were calculated at various times. The apparent first-order constant, λ, and the apparent second-order rate constant for formation of the enzyme-inhibitor complex,

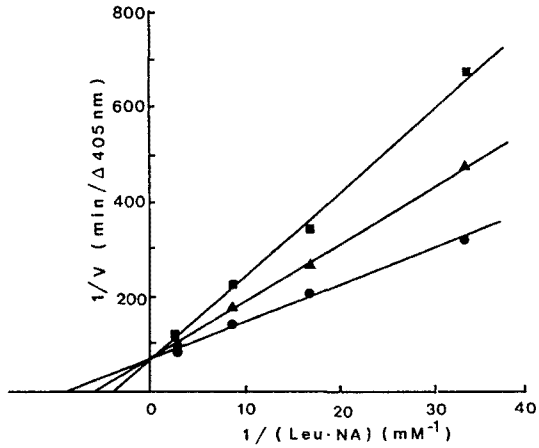

Fig. 5. Lineweaver-Burk plot of inhibition of aminopeptidase N by MR-387A.
●, [I]=0 nM; ▲, [I]=20 nM; ■, [I]=100 nM.

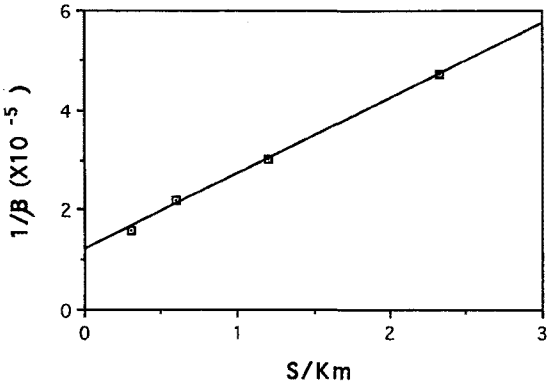

Fig. 6. Replot of the reciprocal second-order rate constant (β) as affected by substrate.
β was determined from the slopes of plots shown in Fig. 4. Concentrations of *p*-nitroaniline liberated by hydrolysis of substrate were determined from the standard curves (y=64.95x-4.95: y, nmole of *p*-nitroanilide; x, O.D. at 405 nm).

β, were determined according to Cha (3). Fitting the data for β in a reciprocal form, $1/\beta=1/k_{on}+S/k_{on}K_m$, indicates a straight-line plot (Fig. 6). The linearity of the 1/β vs S/K_m plot indicates that MR-387A is a competitive inhibitor. From the data of Fig. 6, k_{on} was calculated to be 7.4×10^4 $M^{-1}s^{-1}$. Since k_{off} is 3.3×10^{-5} s^{-1} as shown in Fig. 3, K_i* is 4.4×10^{-10} M from the ratio of k_{off}/k_{on}. These data (K_i and K_i*, see Table 1) indicate that MR-387A and AP-N are bound approximately 200-fold (from K_i/K_i*) more tightly in the final EI* complex than in the initial collision complex.

Rich *et al.* (10) suggested that tri- or tetrapeptide derivatives are better inhibitors of AP-N than are dipeptide derivatives as a consequence of a slower binding process, and that 2(*S*)-hydroxyl group contributes to the stabilization of a collision (EI) complex, which is formed rapidly. Our results obtained from the kinetic studies suggest that novel tetrapeptide MR-387A having a 2(*S*)-hydroxyl group in the structure is a better inhibitor of AP-N with a similar binding mechanism to amastatin.

Table 1. Kinetic parameters for the slow and tight binding of MR-387A to aminopeptidase N.

k_{on}	7.4×10^4 $M^{-1}s^{-1}$
k_{off}	3.3×10^{-5} s^{-1}
K_i* (k_{off}/k_{on})	4.4×10^{-10} M
K_i (Lineweaver-Burk plot)	2.2×10^{-8} M

REFERENCES

1. Aoyagi, T., H. Tobe, F. Kojima, M. Hamada, T. Takeuchi, and H. Umezawa. 1978. Amastatin, an inhibitor of aminopeptidase A, produced by actinomycetes. *J. Antibiot.* **31**: 636-638.
2. Baici, A. 1981. The specific velocity plot. A graphical methods for determining inhibition parameters for both linear and hyperbolic enzyme inhibitors. *Eur. J. Biochem.* **119**: 9-14.
3. Cha, S. 1976. Tight-binding inhibitors III. A new approach for the determination of competition between tight-binding inhibitors and substrates: inhibition of adenosine deaminase by coformycine. *Biochem. Pharmacol.* **25**: 2695-2702.
4. Chung, M. C., H. K. Chun, K. H. Han, H. J. Lee, C. H. Lee, and Y. H. Kho. 1996. MR-387A and B, new aminopeptidase N inhibitors, produced by *Streptomyces neyagawaensis* SL-387. *J. Antibiot.* **49**: 99-102.
5. Chung, M. C., H. K. Chun, H. J. Lee, C. H. Lee, S. I. Kim, and Y. H. Kho. 1996. Inhibition of aminopeptidase N by two synthetic tripeptides. *J. Microbiol. Biotechnol.* **6**: 7-11.
6. Chung, M. C., H. K. Chun, H. J. Lee, and Y. H. Kho. 1994. Taxonomic characteristics of strain producing MR-387A and B, new inhibitors of aminopeptidase M, and their production. *Kor. J. Appl. Microbiol. Biotechnol.* **22**: 447-452.
7. Gros, C., B. Giros, and J. C. Schwartz. 1985. Identification of aminopeptidase M as an enkephaline-inactivating enzyme in rat cerebral membranes. *Biochemistry* **24**: 2179-2185.
8. Gutfreund, H. 1974. Kinetic analysis of the properties and reactions of enzymes. *Prog. Biophys. Mol. Biol.* **29**: 161-195.
9. Helene, A., A. Beaumont, and B. P. Roques. 1991. Functional residues at the active site of aminopeptidase N. *Eur. J. Biochem.* **196**: 385-393.
10. Rich, D. H., B. J. Moon, and S. Harbeson. 1984. Inhibition of aminopeptidases by amastatin and bestatin derivatives. Effect of inhibitor structure on slow-binding processes. *J. Med. Chem.* **27**: 417-422.
11. Schloss, J. V. and W. W. Cleland. 1982. Inhibition of isocitrate lyase by 3-nitropropionate, a reaction-intermediate analogue. *Biochemistry* **21**: 4420-4427.
12. Taylor, A., C. Z. Peltier, F. J. Torre, and N. Hakamian. 1993. Inhibition of bovine lens aminopeptidase by bestatin:

number of binding sites and slow binding of this inhibitor. *Biochemistry* **32**: 784-790.
13. Taylor, A. 1993. Aminopeptidases: towards a mechanism of action. *TIBS* **18**: 167-172.
14. Umezawa, H., T. Aoyagi, T. Tanaka, H. Suda, and T. Takeuchi. 1976. Bestatin, an inhibitor of aminopeptidase B, produced by actinomycetes. *J. Antibiot.* **29**: 97-99.
15. Wilkes, S. H. and J. M. Prescott. 1985. The slow, tight binding of bestatin and amastatin to aminopeptidases. *J. Biol. Chem.* **260**: 13154-13162.
16. Williams, J. W. and J. F. Morrison. 1979. The kinetics of reversible tight-binding inhibition. *Methods Enzymol.* **63**: 437-467.

(Received March 27, 1996)