The role of histidine 231 in thermolysin-like enzymes

Ann Beaumont, Michael O'Donohue, Nathalie Paredes, Nathalie Rousselet, Marcel Assicot, Claude Bohuon, Marie-Claude Fournié-Zaluski, Bernard P. Roques

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 270, No. 28, Issue of July 14, pp. 16803–16808, 1995
Printed in U.S.A.

The Role of Histidine 231 in Thermolysin-like Enzymes

A SITE-DIRECTED MUTAGENESIS STUDY*

(Received for publication, February 24, 1995, and in revised form, May 5, 1995)

Ann Beaumont‡, Michael J. O'Donohue‡§, Nathalie Paredes‡, Nathalie Rousselet‡, Marcel Assicot¶, Claude Bohuon¶, Marie-Claude Fournié-Zaluski‡, and Bernard P. Roques‡‖

From the ‡Département de Pharmacochimie Moléculaire et Structurale, U266 INSERM, URA D1500 CNRS, UFR des Sciences Pharmaceutiques et Biologiques, Université René Descartes, Faculté de Pharmacie, 4, avenue de l'Observatoire, 75270 Paris Cedex 06, France and the ¶Département de Biologie Clinique, Institut Gustave-Roussy, 39, rue Camille Desmoulins, 94805 Villejuif Cedex, France

In the zinc metallopeptidases produced by the genus *Bacillus*, an active site histidine has been proposed to either stabilize the transition state in catalysis by donating a hydrogen bond to the hydrated peptide (Matthews, B. W. (1988) *Acc. Chem. Res.* 21, 333–340) or to polarize a water molecule, which subsequently attacks the peptidyl bond (Mock, W. L., and Aksamawati, M. (1994) *Biochem. J.* 302, 57–68). Site-directed mutagenesis techniques have been used to change this residue in the zinc endopeptidase from *Bacillus stearothermophillus* to either phenylalanine or alanine. At pH 7.0, the k_{cat}/K_m values of the substrate leucine enkephalin for the phenylalanine and alanine mutants were reduced by factors of 430- and 500-fold, respectively, as compared with the wild-type enzyme, mostly due to changes in k_{cat}. In addition, the enzymatic activities of the mutant enzymes showed little pH dependence in the alkaline range, unlike the wild-type enzyme. The mutations did not greatly alter the binding affinities of inhibitors containing sulfydryl groups to chelate the active site zinc, while those of inhibitors containing hydroxamate or carboxylate zinc-chelating groups were increased between 80- and 250-fold. The largest change in the binding affinity of an inhibitor (>5 orders of magnitude) was found with the proposed transition state mimic, phosphoramidon. The results are generally in agreement with x-ray crystallography studies and favor the involvement of the active site histidine in transition state binding.

The closely related extracellular zinc endopeptidases (EC 3.4.24.27) produced by members of the bacterial genus *Bacillus* are thermostable, calcium-binding neutral proteases. Two of these enzymes, from *Bacillus thermoproteolyticus* (thermolysin) and *Bacillus cereus*, have been crystallized to a high resolution and shown to have virtually identical three-dimensional structures and active site geometries (1). From molecular modelling studies, similar structures for other enzymes of the same group have been predicted, such as those secreted by *Bacillus stearothermophillus* (2), *Bacillus subtilis* (3, 4), and *B. subtilis* var. *amylosaccharititicus* (5). An active site model of the neutral proteases, derived from x-ray crystallographic studies of the binding of a variety of different inhibitors to thermolysin (for review, see Ref. 6), has found a wide application, as many mammalian zinc metallopeptidases, such as neutral endopeptidase 24.11 (EC 3.4.24.11) and angiotensin-converting enzyme (EC 3.4.15.1), appear to have thermolysin-like active sites. The model has therefore formed the basis for the design of inhibitors of these physiologically important enzymes (for review, see Ref. 7).

The bacterial enzymes are bilobal, and the active site, situated in a cleft between the two lobes, contains an atom of zinc bound with a tetracoordinate geometry to two histidines and a glutamate, with a water molecule providing the fourth ligand. The two histidines are found in a consensus sequence, HEXXH, present in the majority of zinc endo- and aminopeptidases (8). A large hydrophobic pocket in the P'_1 position confers a preference for cleaving peptides at the N-terminal side of hydrophobic residues, and from the x-ray data it has been deduced that catalysis proceeds by a general base-type mechanism, with the zinc-bound water molecule being displaced toward the glutamate of the consensus sequence (Glu-143)[1] by the incoming substrate. The resulting polarization of this water molecule then leads to an attack of the carbonyl carbon of the scissile bond, with the negative charge that develops on the carbonyl group being stabilized by hydrogen bonds formed with both His-231 and Tyr-157 (Fig. 1*A*) (6). However, an alternative catalytic mechanism has recently been proposed, based on kinetic studies, in which His-231 acts initially as the general base that polarizes the attacking water molecule, with Glu-143 possibly playing a role in charge neutralization (Fig. 1*B*) (9).

The neutral protease produced by *B. stearothermophillus* (NPr)[2] has an 85% sequence homology with thermolysin, which includes all of the important active site residues and, as discussed above, has been predicted to have a similar structure and active site geometry. In this study, site-directed mutagenesis has been used to change the active site histidine (His-231) in this enzyme to either Phe or Ala. Wild-type and mutant enzymes were purified, and their kinetic and inhibitor binding properties were analyzed.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Present address: Laboratoire d'Etude des Protéines, INRA, Centre de Versailles, Route de St. Cyr, 78026 Versailles Cedex, France.

‖ To whom correspondence should be addressed.

[1] The amino acid numbering used throughout the article is that of thermolysin.

[2] The abbreviations and trivial names used are: NPr, neutral protease; *npr*, neutral protease gene; thiorphan, *N*-[2-(*R,S*)-(mercaptomethyl)-1-oxo-3-phenylpropyl]glycine; retrothiorphan, 3-[[1-(*R,S*)-(mercaptomethyl)-2-oxo-3-phenylethyl]amino]-3-oxopropanoic acid; JCH 27, *N*-[2-mercapto-1-oxoheptyl]-L-Phe-L-Ala; HACBOGly, *N*-(2*R*,2*S*)-3-hydroxyaminocarbonyl-2-benzyl-1-oxopropylglycine; ST 51, 2-[(4-hydroxy)-phenylmethyl]-4-*N*-[3-hydroxyamino-3-oxo-1-(phenylmethyl)propyl] amino-4-oxobutanoic acid; ES 92, *N*-[1-carboxy-2-phenylethyl]-L-Phe-L-Leu; phosphoramidon, *N*-[α-rhamnopyransoyl-(oxyhydroxyphosphinyl)]-L-leucyl-L-tryptophan; PAGE, polyacrylamide gel electrophoresis; MES, 2-(*N*-morpholino)ethanesulfonic acid.

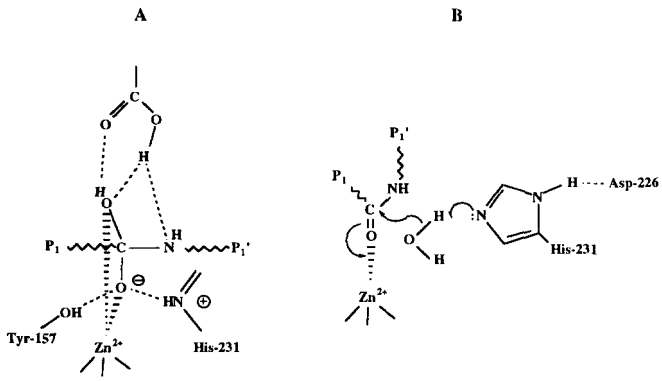


FIG. 1. **Proposed roles of His-231 in NPr catalysis.** *A*, in stabilizing the transition state (adapted from Matthews (6)); *B*, in polarizing a water molecule that would attack the carbon of the peptide bond leading to the formation of the peptide bond (adapted from Mock and Aksamawati (9)). Presumed hydrogen bonds are shown as *broken lines*, and zinc interactions as *striated lines*.

EXPERIMENTAL PROCEDURES

Materials—All culture media components were Difco products obtained from OSI (Maurepas, France). Oligonucleotides were purchased from the laboratory of Professor J. Igolen (Institut Pasteur, Paris, France). The Sequenase™ enzyme (version 2) (U. S. Biochemical Corp.) and [α-^{33}P]dATP were purchased from Amersham Corp. [^{3}H]Tyrosyl-glycyl-glycyl-phenylalanyl-leucine ([^{3}H]leucine enkephalin) was from DuPont NEN. Leucine enkephalin (tyrosyl-glycyl-glycyl-phenylalanyl-leucine) and phenylalanyl-alanine were purchased from Bachem (Bubendorf, Switzerland), and phosphoramidon and thermolysin were obtained from Sigma. The inhibitors, thiorphan (10), retrothiorphan (11), HACBOGly (12), and ST 51 (13), were synthesized in the laboratory as described previously. The synthesis of the other inhibitors, JCH 24 and ES 92 will be published elsewhere. The structures of these inhibitors are given (see Table II). Poropak Q was from Waters (Saint-Quentin Fallavier, France). Other chemicals were purchased from Sigma or Prolabo (Paris, France).

Bacterial Strains, Plasmids, Bacteriophage, and Culture Media—The *Bacillus* strain used throughout was *B. subtilis* DB117, lacking neutral protease activity (Em^r *his nprR2 nprE18 spr A3, npr*$^-$) (3). For site-directed mutagenesis experiments, the *Escherichia coli* strains, WK-6 *muts* and WK-6, were employed. Npr was produced using the plasmid, pGE501(Cm^r), which contains the entire coding sequence of the neutral protease (3). In order to mutate the *npr* gene, the plasmid pMa (14) containing the Mlu 1 fragment of pGE501 (pMaMlu) was employed, and to reconstitute the *npr* gene, the mutated fragment was ligated into the unique Mlu 1 site of pGE501 Mlu, which is a derivative of pGE501 in which the Mlu 1 fragment has been deleted. *B. subtilis* DB117 was propagated at 37 °C in LB broth or on tryptose blood agar base plates, containing 5 μg/ml erythromycin. *B. subtilis* DB117 containing pGE501, or one of its derivatives, was grown in the same media containing 5 μg/ml chloramphenicol. In order to screen for protease activity by colony halo formation on Petri dishes, skimmed milk (1% (w/v)) was included in these media, solidified with 1.5% (w/v) agar. *B. subtilis* protoplasts were regenerated on DM3 plates (15). *E. coli* were grown routinely on LB broth at 37 °C with antibiotics, ampicillin, or chloramphenicol being added to final concentrations of 100 and 25 mg/ml, respectively, when required.

Plasmid DNA Isolation and Manipulation—Plasmid DNA was isolated from *B. subtilis* by a modified form of the alkaline lysis method described by Bron (16). Further purification of large scale plasmid preparations was achieved using a modified polyethylene glycol precipitation method (17). Extraction and purification of double-stranded pMaMlu DNA was performed using a Quiagen kit. For the production of single-stranded pMa plasmids, the helper bacteriophage M13K07 was used, and single-stranded DNA was subsequently extracted using standard methods. DNA sequencing of double-stranded plasmid DNA was performed using the dideoxynucleotide termination method (18) using the Sequenase™ enzyme (version 2), [α-^{33}P]dATP, and oligonucleotides complementary either to parts of the pMa/c vector or to the NprSte gene.

Transformation of B. subtilis DB117—For plasmid transformation, *B. subtilis* protoplasts were prepared as described by Bron (15).

Site-directed Mutagenesis—Mutagenesis was performed using the gapped duplex method (14) in which mutations are accompanied by a switch in antibiotic resistance. The following oligonucleotides were employed to mutate His-231 to either Ala or Phe: H231A, 5′ GCT GTT TGT AGC GAC GCC GCC 3′; and H234F, 5′ GCT GTT TGT AAA GAC GCC GCC 3′.

The mutated codon is underlined. Mutated pMaMlu were identified by DNA sequencing, and mutated Mlu 1 fragments were ligated into the plasmid pGB501 Mlu; the ligation mixture being used to directly transform *Bacillus* protoplasts. Subsequently, halo-producing clones were identified and further analyzed by restriction digest analysis and DNA sequencing.

Expression and Purification of the Extracellular Proteases—B. subtilis DB117 cells harboring the plasmid pGE501 were grown in LB broth containing 5 mM $CaCl_2$ and 5 μg/ml chloramphenicol, at 37 °C, with shaking (180 rpm) for 16 h. Wild-type and mutated enzymes were purified from the culture supernatant by affinity chromatography using a column (1 × 4 cm) of Gly-D-Phe, coupled to CNBr-activated Sepharose 4B resin following the manufacturer's instructions and equilibrated in 20 mM sodium acetate, pH 5.5, containing 5 mM $CaCl_2$. The culture supernatant, obtained by centrifugation of the culture at 6000 × *g* for 10 min, was slowly adjusted to pH 5.5 and passed over the column at a flow rate of 3 ml/h. The column was then washed with the equilibration buffer followed by the equilibration buffer containing 1 M NaCl, and the enzymes were finally eluted with 20 mM sodium acetate buffer, pH 5.5, containing 5 mM $CaCl_2$, 2.5 M NaCl, and 20% (v/v) propan-2-ol. All enzyme activities were stable in this buffer for several months when stored at −20 °C. Enzyme purity was checked by SDS-PAGE using 12% slab gels. Protein concentration was determined following the method of Bradford (19).

Assay for Enzymatic Activity—Enzymatic activity was normally assayed at 37 °C in a total volume of 100 μl of 50 mM HEPES, pH 7. 0, containing 5 mM $CaCl_2$, with 50 nM [^{3}H]leucine enkephalin as a substrate (20). Reactions were stopped by the addition of 10 μl of 0.5 M HCl and the metabolite, [^{3}H]Tyr-Gly-Gly, was separated for liquid scintillation counting, using columns of Poropak Q as described previously (21). Inhibitors, at varying concentrations, were preincubated with the enzymes for 30 min before the addition of substrate, and as the concentration of substrate used for inhibition and pH studies was less than its K_m for the enzyme, IC_{50} values were taken to be equal to K_i values (22). For pH studies, the buffers were used at 20 mM with the ionic strength kept at 0.05 with NaCl. K_m and k_{cat} values for leucine enkephalin degradation were determined using the substrate over a concentration range of 0.05–4 mM, with 25 nM [^{3}H]leucine enkephalin included as a tracer. The values were calculated by linear regression analysis using the program Enzfit (Biosoft). The change in the free energies of binding were calculated from $\Delta G = RT\ln((k_{cat}/K_m)\text{wt}/(k_{cat}/K_m)\text{mut})$ or $\Delta G = RT\ln(K_i\text{wt}/K_i\text{mut})$. In all cases, reactions were stopped when substrate degradation was ≤10%.

SDS-PAGE and Western Blots—SDS-PAGE was carried out using 12% polyacrylamide slab gels. Monoclonal antibodies were raised against commercially available thermolysin, and one, monoclonal antibody T1, was found, in enzyme-linked immunosorbent assays, to also cross-react with NPr. Mapping experiments (not shown) showed that monoclonal antibody T1 recognizes an epitope contained within residues 206–255 of thermolysin, a region where there are only two amino acid differences in the sequences of thermolysin and NPr (2). For Western blots, 1 ml of the culture was centrifuged at 12,000 × *g*, and 5 μl of the supernatant were mixed with 15 μl of SDS-PAGE sample buffer. For intracellular extracts, the pellet was resuspended in 100 μl of 50 mM Tris-HCl, pH 7.2, containing 15% (w/v) sucrose and 1 mg/ml lysozyme. After a 5-min incubation at 37 °C, 180 μl of sample buffer was added, and 10 μl were taken for SDS-PAGE. Electroblotting, to nitrocellulose sheets, was carried out for 2 h at 200 V, and the blots were developed using a streptavidine-alkaline phosphatase system (Amersham Corp.).

RESULTS AND DISCUSSION

Expression and Purification of the Wild-type and Mutated Enzymes

Wild-type and mutant NPrs were purified in a single step using the Gly-D-Phe affinity column with yields of around 5 mg/liter for the wild-type enzyme and 0.5 mg/liter for H231F-NPr and H231A-NPr. SDS-PAGE showed, for the three enzymes, the presence of a single protein with an apparent molecular mass of 36 kDa (Fig. 2). Recovery in each case was estimated to be around 50%, and the reduced yields of the mutant enzymes are therefore likely to be due to a decrease in

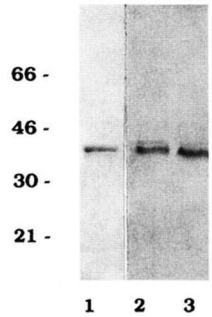


FIG. 2. **SDS-PAGE (12%) of purified wild-type and mutant enzymes.** *Lane 1*, 3 μg of wild-type NPr; *lane 2*, 5 μg of H231F-NPr; *lane 3*, 5 μg of H231A-NPr.

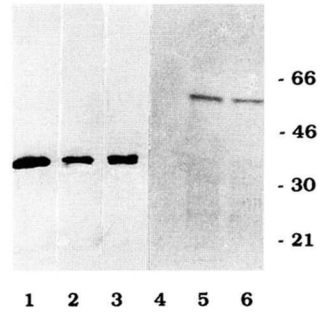


FIG. 3. **Western blots of extracellular and total cell extracts of *B. subtilis*.** Extracellular extracts were as follows: *lane 1*, wild-type NPr; *lane 2*, H231F-NPr; *lane 3*, H231A-NPr. Total cell extracts were as follows: *lane 4*, wild-type NPr; *lane 5*, H231F-NPr; *lane 6*, H231A-NPr.

the extracellular levels of the enzymes. The *Bacillus* neutral proteases are synthesized as preproenzymes, with the prosequence (200 residues for NPr) probably being removed at the cell membrane (23). This is thought to be an autocatalytic process, and mutations that change enzyme activity might therefore be expected to affect the levels of mature enzyme produced. This appeared to be the case for the mutations H231F and H231A, as Western blot analysis of the culture supernatants, using the monoclonal antibody Mab T1, showed that the wild-type enzyme was present in higher levels (Fig. 3). In contrast, in total cell extracts, a 60-kDa protein was detected, only in *B. subtilis* transformed with the mutated enzymes, which could correspond to the unprocessed proenzyme. Similar results were obtained when the active site histidine of the neutral protease from *B. subtilis* was mutated (24).

Enzymatic Activity of Wild-type and Mutant Enzymes

At pH 7.0 in HEPES buffer, the K_m values of the substrate, leucine enkephalin for H231F and H231A-NPr, were almost identical to its K_m for the wild-type enzyme, indicating that mutating His-231 does not provoke any major changes in ground state binding. For the mutant enzymes, however, k_{cat} values were lower, leading to decreases in k_{cat}/K_m of 430- and 500-fold for H231F and H231A, respectively (Table I) equivalent to a change in ΔG of 3.7–3.8 kcal/mol. These results are comparable with those obtained with the zinc exopeptidase, carboxypeptidase A (EC 3.4.17.1), whose active site has a similar topology to thermolysin (6, 25). A similar mechanism of action has been proposed for both enzymes, with Arg-127, corresponding to His-231 of NPr, stabilizing the transition state, and site-directed mutagenesis of this residue led to large reductions in k_{cat} with relatively small changes in K_m (26). In this instance, it was calculated that the arginine could stabilize the transition state by 4.1–6.0 kcal/mol. Another zinc endopeptidase, neutral endopeptidase 24.11 (EC 3.4.24.11) is also thought to have an active site organization that closely resembles that of the *Bacillus* enzymes, although no crystal structure is available (for review, see Ref. 7). In this enzyme, His-711 has been proposed to stabilize the transition state (27), and mutating this residue to Phe led to a 40-fold reduction in k_{cat}/K_m, lower than found here, but the changes were again primarily due to a decrease in k_{cat} (28), indicating that His-711 of the mammalian enzyme and His-231 of the *Bacillus* enzymes may have similar roles in their respective enzymes.

TABLE I
Kinetic constants for the hydrolysis of leucine enkephalin by wild-type, H231F, and H231A NPrs

Reactions were carried out in 50 mM HEPES buffer pH 7.0 as described under "Experimental Procedures."

Enzyme	K_m	k_{cat}	k_{cat}/K_m
	μM	$M^{-1} \cdot s^{-1}$	s^{-1}
Wild-type	140 ± 25	180 ± 17.1	1.29×10^6
H231F	205 ± 30	0.63 ± 0.11	3.07×10^3
H231A	127 ± 15	0.33 ± 0.06	2.56×10^3

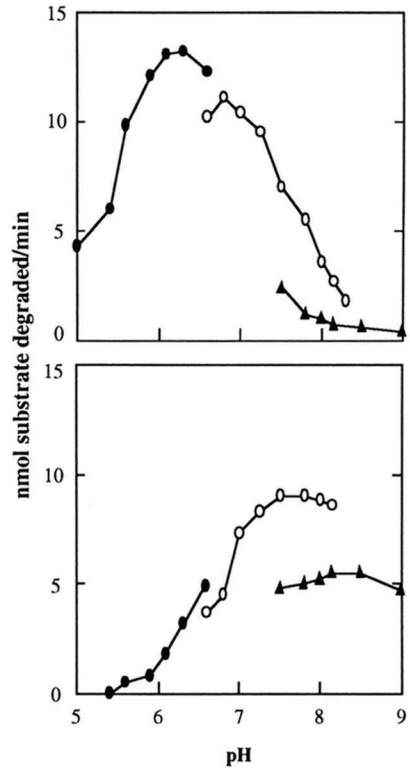
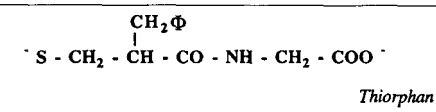
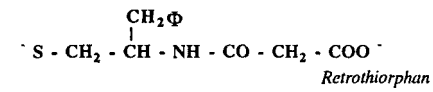
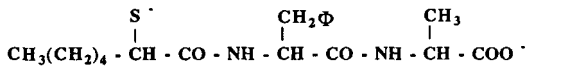
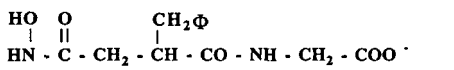
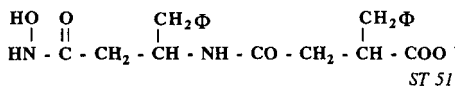
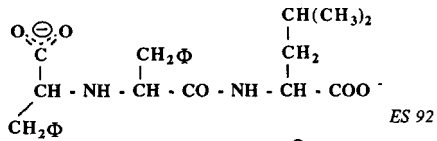
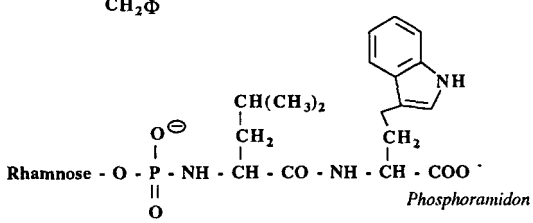


FIG. 4. **Activity of 0.2 ng wild-type NPr (*top*) and 50 ng of H231F-NPr (*bottom*) with pH.** The assay was carried out at constant ionic strength, using 50 nM [^{3}H]leucine enkephalin as substrate as described under "Experimental Procedures." The buffers used were MES (●), HEPES, (○), and Tris (▲).

PH Optimum

The optimum pH for the degradation of 50 nM [^{3}H]leucine enkephalin by the wild-type enzyme was in the range of pH 6.0–6.8 (Fig. 4), and, as previously reported for thermolysin (29), enzymatic activity varied with the buffer used, being higher in HEPES than in Tris. For the mutant enzyme H231F-NPr, optimum activity was between pH 7.5 and 8.5, and at pH 8.5 in Tris buffer the mutant enzyme was only 30-fold less active than the wild-type enzyme. The activity of the *Bacillus* neutral proteases has been shown to depend on two groups, one with an acidic pK_a, suggested to be either Glu-143 (6) or the zinc-bound water molecule (9), and a second species with a pK_a

TABLE II
K_i values of inhibitors, for wild-type and mutated NPrs

Inhibitors	K_i (μM)		
	Wild type	H231 F	H231 A
 *Thiorphan*	1.5 ± 0.1	2.25 ± 0.21	2.5 ± 0.1
 *Retrothiorphan*	0.8 ± 0.1	1.11 ± 0.12	1.55 ± 0.15
 *JCH 27*	0.06 ± 0.01	0.07 ± 0.01	0.15 ± 0.04
 *HACBO-Gly*	3.56 ± 0.21	290 ± 16	285 ± 25
 *ST 51*	0.19 ± 0.03	35 ± 5	19 ± 3
 *ES 92*	2.2 ± 0.2	510 ± 21	540 ± 26
 *Phosphoramidon*	0.063 ± 0.006	>1,000	>1,000

around 7.5–8.0, suggested to be His-231 (6, 9). As expected, therefore, little pH dependence was observed in the alkaline pH range with H231F-NPr, and the pK_a of the remaining ionizable group was also apparently influenced by the mutation.

Inhibition of Wild-type and Mutant Enzymes

The inhibition of [^{3}H]leucine enkephalin hydrolysis by wild-type and mutated enzymes was compared using representative molecules of the different classes of inhibitors for neutral proteases (Table II). The presumed binding modes of these molecules to the active site of NPr are shown in Fig. 5, taken from data obtained by the cocrystallization of these molecules or their analogues with thermolysin (6, 30). All of the molecules tested have a hydrophobic residue to fit into the S'$_1$ subsite and a strong zinc-chelating group. It is important to note that the K_i values of the inhibitors for the wild-type NPr were not significantly different to those obtained with thermolysin (31),[3] confirming the similarities in the active sites of the two proteases. The values reported in Table II were obtained using 50 mM HEPES at pH 7.0 containing 5 mM $CaCl_2$.

Thiol Inhibitors—Mutating His-231 to either Phe or Ala did not modify significantly the K_i values of the sulfydryl-containing inhibitors thiorphan, retrothiorphan, and JCH 24. This is in agreement with molecular modelling studies (20) and the cocrystallization of thiorphan and retrothiorphan with thermolysin (30), showing that these molecules bind as substrate analogues, with no apparent interaction with His-231, as previously reported with another thiorphan-like inhibitor, (2-benzyl-3-mercaptopropanoyl)-L-alanylglycinamide (32). It was found that, as predicted (20), both thiorphan and retrothiorphan have similar interactions in the active site despite the retroinversion of the amide bond in the latter. The most important of these are the coordination of the SH group with the zinc atom, the binding of the P'$_1$ residue in the S'$_1$ subsite, and hydrogen bonding between the carbonyl and carbonyl amide group of the peptide bond with Arg-203 and Asn-113, respectively. The fact that mutating His-231 made little difference to the K_i values of these inhibitors indicates that these interactions can still be fulfilled and again shows that the overall geometry of the active site in its ground state has probably not been greatly altered. Again these results can be compared with those found for carboxypeptidase A, where mutating Arg-127 had only a small effect on the binding of ground state inhibitors (26).

Hydroxamate and Carboxylate Inhibitors—Mutating His-231 decreased significantly the affinities of the hydroxamate inhibitors HACBOGly and ST 51, and the carboxylate inhibitor ES 92. For HACBOGly, K_i values were increased 80-fold, and for ST 51 the increases were 100-fold for H231F-NPr and 180-fold for H231A-NPr, equivalent to a change in ΔG of between 2.7 and 3.2 kcal/mol. Similar hydroxamate inhibitors have been described as mimicking reaction intermediates,

[3] A. Beaumont, M. O'Donohue, N. Paredes, N. Rousselet, M. Assicot, C. Bohuon, M.-C. Fournié-Zaluski, and B. P. Roques, unpublished observations.

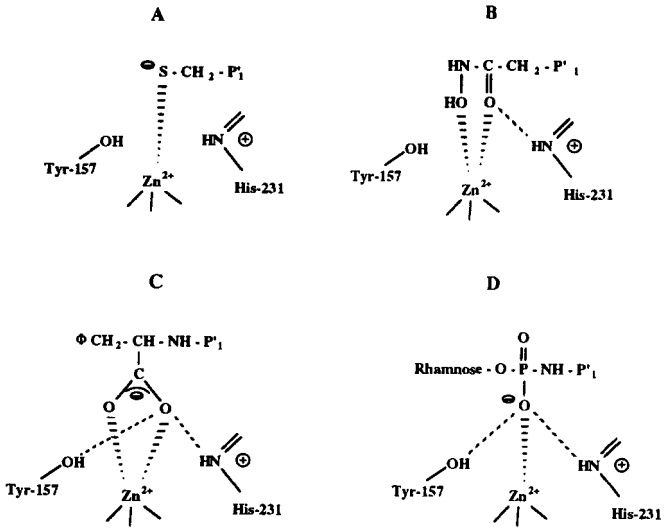


FIG. 5. **Presumed binding modes of the inhibitors used in this study to the active site of NPr.** Only the expected interactions with the zinc atom (*striated lines*) and His-231 and Tyr-157 (*dotted lines*) are shown. *A*, thiol inhibitors (30, 32); *B*, hydroxamate inhibitors (33); *C*, carboxylate inhibitors (25, 34); *D*, phosphoramidon (35).

binding to the active site of thermolysin in a bidentate fashion, with the carbonyl oxygen and the hydroxyl oxygen of the hydroxamate group ligated to the zinc (33), resembling the presumed pentacoordinate complex of the transition state in catalysis (6). The formation of a hydrogen bond between the carbonyl oxygen of the bidentate group and His-231 was also predicted. With the carboxylate inhibitor ES 92, mutating His-231 provoked a 250-fold increase in K_i or a change in ΔG of 3.4 kcal/mol. Again, this is in line with x-ray crystallographic studies of thermolysin, showing that the bidentate mode of binding of a carboxylate group resembles the presumed geometry of the tetrahedral transition state with a hydrogen bond being formed between one oxygen of the carboxyl group and His-231 (25, 34).

Phosphoramidon—The largest differences in K_i values were found with the phosphoramidate inhibitor, phosphoramidon, which had a K_i of 63 nM for the wild-type enzyme, with no inhibition of either H231F or H231A-NPr being observed at an inhibitor concentration of 1 mM, giving a difference in binding energy of >6 kcal/mol. Such a large increase in K_i was perhaps unexpected, although phosphoramidon has been described as a transition state inhibitor with one of its phosphoryl oxygens ligated to the zinc in a tetrahedral complex and also forming hydrogen bonds with His-231 and Tyr-157 (6, 35). In addition, the binding of phosphoramidon in the alkaline range has been shown to be critically dependent on one ionizable group (36), which is likely to be His-231. Moreover, mutating Arg-127 of carboxypeptidase A also led to large decreases in the binding affinity of a transition state analogue of 5.8 kcal/mol (26).

Conclusions

Structural studies have indicated that His-231 of the *Bacillus* neutral proteases stabilizes the transition state by forming a hydrogen bond with the oxygen of the amide bond to be cleaved (Fig. 1*A*) (6). On the other hand, a more crucial function for the residue has recently been proposed from mechanistic studies, in which it would polarize the attacking water molecule in the first step of catalysis (Fig. 1*B*) (9), a role previously subscribed to Glu-143 (6). The results presented here would seem to be in favor of the less critical role for His-231 in catalysis, as its replacement by either Phe or Ala led to the production of enzymes that still retained proteolytic activity, albeit reduced. In addition, this reduction, mostly due to a decreased k_{cat}, was of the same order as that obtained when the residue proposed to stabilize the transition state of carboxypeptidase A (Arg-127) was mutated (26). This is in contrast to mutations of Glu-143 from the neutral proteases of *B. stearothermophillus* MK232 (37) and *B. subtilis* (24), which led to enzymes with undetectable proteolytic activity. Similar results were obtained when the corresponding consensus sequence glutamate of neutral endopeptidase 24.11 was mutated (38). It was suggested that loss of activity provoked by these mutations might be due to major perturbations of the active site, resulting from the replacement of the negatively charged Glu (9). However, inactive mutants were obtained even when the Glu was changed to Asp (37, 38), and, in addition, inhibitor binding to the Asp mutant of neutral endopeptidase-24.11 was unchanged (38).

Further evidence for a nonessential role of His-231 has come from x-ray crystallographic studies of representative members of the metzincin family of zinc endopeptidases (39) such as astacin (40, 41), matrix metalloproteases (42, 43, 44), and adamalysin II (45), in which no equivalent residue has been found. These enzymes differ from the thermolysin family in that the three zinc-binding ligands are histidines contained in an extended consensus sequence, HE*XX*H*XX*G*XX*H. Despite this difference, a close topology has been found with thermolysin in the zinc-binding environment, and it is likely that the two families have similar mechanisms of action (41, 44). It has been proposed that the zinc in the metzincins would have a higher net positive charge than in thermolysin, where one ligand is a negatively charged carboxylate, allowing it to play a larger role in stabilizing the negative charge of the transition state and obviating the need for an equivalent to His-231 (43, 44). In addition, as with the thermolysin-like enzymes, mutation of the consensus sequence glutamate in one of the metzincins, endopeptidase 24.18, led to a total loss of detectable enzyme activity (46).

The results of the inhibition studies are also in agreement with x-ray crystallographic studies of thermolysin. In the thiol-containing inhibitors crystallized with the enzyme, the distance between the sulfur atom and His-231 as well as Tyr-157 was found to be too far for hydrogen bonding (6, 30), and the absence of such an interaction with His-231 has been confirmed by the lack of change in the K_i values of three thiol inhibitors for the mutated enzymes. Nevertheless, in spite of the lack of these stabilizing interactions, such molecules can be potent inhibitors whatever the type of zinc metallopeptidase studied (7, 47). This could be due to favorable interactions of electrons in the external *d* orbital of sulfur with the zinc cation, which does not impose a strict directionality. For the hydroxamate and carboxylate molecules studied here, an interaction with His-231 would be expected from x-ray crystallographic data (6, 33, 34), and this again was reflected in the results obtained, with increases in K_i values ranging from 80- to 250-fold for the mutated enzymes.

Again, for the binding of phosphoramidon, an interaction with His-231 would be expected from crystallographic studies (6, 35). However, for this inhibitor, the presence of the residue seems to be even more critical. It is unlikely that inhibitor binding was prevented by any direct compression around the zinc due to the mutation, as there was a total loss of measurable binding affinity for both the Phe and Ala mutants. Several hypotheses can be proposed to account for the loss of affinity observed. The formation of a hydrogen bond with His-231, and possibly Tyr-157, might be required to limit the rotation of the P–N–C bonds, allowing optimal interaction between the oxygen atom of the phosphoramide group and the zinc cation. Alternatively, the possible loss of a structured water molecule(s) pre-

sent in the wild-type enzyme could be unfavorable for binding to the mutant enzymes, or, conversely, the presence of additional unstructured water molecule(s) in the active sites of the mutated enzymes could have a similar effect. Further studies, including those of molecular modelling, will be required to ascertain the exact role of His-231 in phosphoramidon binding.

Acknowledgments—We thank Dr. C. Vita (CEA-Saclay-France) for providing some of the thermolysin peptides used in antibody mapping and C. Dupuis for invaluable help in preparing this manuscript.

REFERENCES

1. Pauptit, R. A., Karlsson, R., Picot, D., Jenkins, J. A., Niklaus-Reimer, A.-S., and Jansonius, J. N. (1988) *J. Mol. Biol.* **199,** 525–537
2. Vriend, G., and Eijsink, V. (1993) *J. Comput. Aided Mol. Des.* **7,** 367–396
3. Eijsink, V. G. H., Vriend, G., van den Burg, B., Venema, G., and Stulp, B. K. (1990) *Protein Eng.* **4,** 99–104
4. Signor, G., Vita, C., Fontana, A., Frigerio, F. Bolognesi, M., Toma, S., Gianna, R., De Gregoriis, E., and Fontana, A. (1990) *Eur. J. Biochem.* **189,** 221–227
5. Tsuru, D., Imajo, S., Morikawa, S.-y., Yoshimoto, T., and Ishiguro, M. (1993) *J. Biochem. (Tokyo)* **113,** 101–105
6. Matthews, B. W. (1988) *Acc. Chem. Res.* **21,** 333–340
7. Roques, B. P., Noble, F., Daugé, V., Fournié-Zaluski, M.-C., and Beaumont, A. (1993) *Pharmacol. Rev.* **45,** 87–146
8. Rawlings, N. D., and Barrett, A. J. (1993) *Biochem. J.* **290,** 205–218
9. Mock, W. L., and Aksamawati, M. (1994) *Biochem. J.* **302,** 57–68
10. Roques, B. P., Fournié-Zaluski, M.-C., Soroca, E., Lecomte, J.-M., Malfroy, B., Llorens, C., and Schwartz, J.-C. (1980) *Nature* **288,** 286–287
11. Roques, B. P., Lucas-Soroca, E., Chaillet, P., Constentin, J., and Fournié-Zaluski, M.-C. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80,** 3178–3182
12. Bouboutou, R., Waksman, G., Devin, J., Fournié-Zaluski, M. C., and Roques, B. P. (1984) *Life Sci.* **35,** 1023–1030
13. Fournié-Zaluski, M. C., Soleihac, J. M., Turcaud, S., Lai-Kuen, R., Crine, P., Beaumont, A., and Roques, B. P. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 6388–6392
14. Stanssens P., Opsomer, C., McKeown, Y. M., Kramer, W., Zabeau, M., and Fritz, H.-J. (1989) *Nucleic Acids Res.* **17,** 4441–4454
15. Bron, S. (1990a) in *Modern Microbiological Methods Series Molecular Biological Methods for Bacillus* (Harwood, C. R., and Cutting, S. M., eds) pp. 142–143, John Wiley and Sons, New York
16. Bron, S. (1990b) in *Modern Microbiological Methods Series: Molecular Biological Methods for Bacillus* (Harwood, C. R., and Cutting, S. M., eds) pp. 154–155, John Wiley & Sons, New York
17. Nicoletti, V. G., and Condorelli, D. F.(1993) *BioTechniques* **14,** 536
18. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74,** 5463–5467
19. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
20. Benchetrit, T., Fournié-Zaluski, M.-C., and Roques, B. P. (1987) *Biochem. Biophys. Res. Commun.* **147,** 1034–1040
21. Vogel, Z., and Altstein, M. (1977) *FEBS Lett.* **80,** 332–35
22. Cheng, Y., and Prusoff, W. H. (1973) *Biochem. Pharmacol.* **22,** 3099–3108
23. Simonen, M., and Palva, I. (1993) *Microbiol. Rev.* **57,** 109–137
24. Toma, S., Campagnoli, S., De Gregoriis, E., Gianna, R., Margarit, I., Zamai, M., and Grandi, G. (1989) *Protein Eng.* **2,** 359–364
25. Hausrath, A. C., and Matthews, B. W. (1994) *J. Biol. Chem.* **269,** 18839–18842
26. Phillips, M. A., Fletterick, R., and Rutter, W. J. (1990) *J. Biol. Chem.* **265,** 20692–20698
27. Bateman, R. C., Jr., Kim, Y.-A., Slaughter, C., and Hersh, L. B. (1990) *J. Biol. Chem.* **265,** 8365–8368
28. Dion, N., LeMoual, H., Crine, P., and Boileau, G. (1993) *FEBS Lett.* **318,** 301–304
29. Feder, J., Brougham, L. R., and Wildi, B. S. (1974) *Biochemistry* **9,** 2784–2791
30. Roderick, S. L., Fournié-Zaluski, M. C., Roques, B. P., and Matthews, B. W. (1989) *Biochemistry* **28,** 1493–1497
31. O'Donohue, M. J., Roques, B. P., and Beaumont, A. (1994) *Biochem. J.* **300,** 599–603
32. Monzingo, A. F., and Matthews, B. (1982) *Biochemistry* **21,** 3390–3394
33. Holmes, M. A., and Matthews, B. W. (1981) *Biochemistry* **20,** 6912–6920
34. Monzingo, A. F., and Matthews, B. W. (1984) *Biochemistry* **23,** 5724–5729
35. Tronrud, D. E., Monzingo, A. F., and Matthews, B. W. (1986) *Eur. J. Biochem.* **157,** 261–268
36. Kitagishi, K., and Hiromi, K. (1984) *J. Biochem. (Tokyo)* **95,** 529–534
37. Kubo, M., Mitsuda, Y., Takagi, M., and Imanaka, T. (1992) *Appl. Environ. Microbiol.* **58,** 3779–3783
38. Devault, A., Nault, C., Zollinger, M., Fournié-Zaluski, M.-C., Roques, B. P., Crine, P., and Boileau, G. (1988) *J. Biol. Chem.* **263,** 4033–4040
39. Bode, W., Gomis-Rüth, F. X., and Stocker, W. (1993) *FEBS Lett.* **331,** 134–140
40. Bode, W., Gomis-Rüth, F. X., Huber, R., Zwilling, R., and Stocker, W. (1992) *Nature* **358,** 164–167
41. Gomez-Rüth, F. X., Stöcker, W., Huber, R., Zwilling, R., and Bode, W. (1993) *J. Mol. Biol.* **229,** 945–964
42. Gooley, P. R., O'Connell, J. F., Marcy, A. I., Cuca, G. C., Salowe, S. P., Bush, B. L., Hermes, J. D., Esser, C. K., Hagmann, W. K., Springer, J. P., and Johnson, B. A. (1994) *Nature Struct. Biol.* **1,** 111–118
43. Lovejoy, B., Cleasby, A., Hassell, A. M., Longley, K., Luther, M. A., Weigl, D., McGeehan, G., McElroy, A. B., Drewry, D., Lambert, M. H., and Jordan, S. R. (1994) *Science* **236,** 375–377
44. Lovejoy, B., Hassell, A. M., Luther, M. A., Weigl, D., and Jordan, S. R. (1994) *Biochemistry* **33,** 8207–8217
45. Gomez-Rüth, F. X., Kress, L. F., Kellerman, J., Mayr, I., Lee, X., Huber, R., and Bode, W. (1994) *J. Mol. Biol.* **239,** 513–544
46. Corbeil, D., Milhiet, P.-M., Simon, V., Ingram, J., Kenny, A. J., Boileau, G., and Crine, P. (1993) *FEBS Lett.* **335,** 361–366
47. Thorsett, E. D., and Wyvratt, M. J. (1987) in *Neuropeptides and their Peptidases* (Turner, A. J., ed.) pp. 229–292, Horwood, New York