Low Barrier Hydrogen Bond Is Absent in the Catalytic Triads in the Ground State but Is Present in a Transition-state Complex in the Prolyl Oligopeptidase Family of Serine Proteases

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Ara Kahyaoglu‡, Khadijeh Haghjoo‡, Fusheng Guo‡, Frank Jordan‡‡, Charles Kettner‡, Ferenc Felföldi‡, László Polgár†

From the ‡Department of Chemistry, Rutgers, the State University of New Jersey, Newark, New Jersey, 07102, the †DuPont Merck Pharmaceutical Company, Wilmington, Delaware 19880-0328, and the ¶Institute for Enzymology, Biological Research Center of the Hungarian Academy of Sciences, Karolina u. 29-31, H-1113, Budapest, Hungary

High frequency proton NMR spectra for two members of the prolyl oligopeptidase class of serine proteases, prolyl oligopeptidase and oligopeptidase B, showed that resonances corresponding to the active center histidine N\(^{13}\)H and N\(^{15}\)H generally observed in this region, are absent in these enzymes. However, for both enzymes, as well as with the H652A and H652Q active center variants of oligopeptidase B, there are two resonances observed in this region that could be assigned to two protonated histidines with a noncatalytic function. The results indicate that these two histidines participate in strong hydrogen bonds. The absence of resonances pertinent to the active center histidine resonances suggests the absence of a low barrier hydrogen bond between the Asp and His in these two enzymes in their ground states. Addition of the peptide boronic acid t-butoxy carbonyl-(D)Val-Leu-(L)boroxArg to oligopeptidase B resulted in potent, slow binding inhibition of the enzyme and the appearance of a new resonance at 15.8 ppm, whose chemical shift is appropriate for a tetrahedral boronate complex and a low barrier hydrogen bond. The results demonstrate important dissimilarities between the active centers of the prolyl oligopeptidase class of serine proteases and the pancreatic and subtilisin classes both in the ground state and in the transition-state analog complexes.

The hydrogen bond between the Asp and His residues in the catalytic triad of serine proteases has been presented as an example of the potential contribution of low barrier hydrogen bonds (LBHB)\(^1\) to catalysis of enzymatic reactions (1–3). It was hypothesized by some that such an LBHB could lead to tran-
presence of strongly hydrogen-bonded non-catalytic histidines in both enzymes.

EXPERIMENTAL PROCEDURES

Proton Nuclear Magnetic Resonance—Proton nuclear magnetic resonance experiments were carried out in Wilmad 535-PP 5-mm tubes at 400 and 500 MHz on a Varian VXR 400–89 instrument at 10 °C. Acquisition time was 0.8 s; delay time = 0.1 s; line broadening 60 Hz; transients recorded: 90,000–120,000; spectral window 14,000 Hz; number of points: 22,400; block size 32. The 1–1 binomial water suppression method was used (36). Backward linear prediction was used to improve the signal to noise, and especially the baseline. From bottom to top: PO at pH 8.22, PO at pH 7.0, PO with Z-Pro-Proline at pH 7.0, and PO with Z-Pro-Proline at pH 5.14 (top).

Prolyl Oligopeptidase—PO was isolated from pig muscle as described (20). The activity of PO was determined fluorimetrically with benzyl-oxycarbonyl-Gly-Pro-2-naphthylamide as substrate (20) by using a Jasco FP777 spectrofluorimeter. The excitation and emission wavelengths were, respectively, 340 and 410 nm.

Oligopeptidase B—OpB was expressed in E. coli JM83 and purified with slight modification as described by Kanatani et al. (23). The activity of the enzyme was measured spectrophotometrically with N-benzoyl-L-Arg-p-nitroanilide-HCl (Sigma) as substrate at 410 nm (ε = 8900 M⁻¹ cm⁻¹, see Ref. 24). The substrate and the enzyme were both dissolved in 0.1 M Tris buffer, pH 8.0, containing 1 mM EDTA.

Preparation of Mutant Oligopeptidase B—The pSKOpB vector contained the wild-type oligopeptidase gene. To change the active site His652 to Gln or Ala, the primers H652Q (GGACTCACCGCCAAGGC- CGGGCAAATCTGG) containing the BglII restriction enzyme site and H652A (GGAACTTCAAGGCGCCGGCGGCAAATCTGG) containing the EheI site were synthesized. The original NcoI site was eliminated in both primers. The desired mutations were introduced with one of the sense primers His652Gln or His652Ala and the antisense PROT3 primer (CGGTCGACGAACCGCGATCCGGGC), with a SalI restriction enzyme site underlined. The PCR-I product was synthesized in a mixture containing the pSKOpB vector as template, 100 nmol each of the two primers, 400 μM dNTP, 0.02 units/ml of Pfu DNA polymerase (Stratagene) in 1/3 Pfu reaction buffer (Stratagene) with 30 cycles at 94, 55, and 72 °C for 1, 2, and 3 min, respectively. The PCR product (200 base pairs) was identified on an agarose gel (1.7%), extracted, and purified on Sephaglas (Pharmacia Biotech Inc.).

The second round of PCR used the same program as in the previous paragraph, using the pSKOpB vector as template with the 200-base pair megaprimer and the PROT5 primer (CGAATTCATCCCCGGTGAGTCTTGGCCACC) containing the underlined EcoRI site (providing the second PCR product). Subcloning was carried out by double digestion of this second PCR product with EcoRI and SalI restriction enzyme and then ligation into the EcoRI/SalI sites of a pBluescript SK(+) vector (Stratagene). The resulting pSKOpBH652Q and...
FIG. 1. Kinetics of inhibition of oligopeptidase B with Boc-(d)Val-Leu-(l)boroArg. A, Lineweaver-Burk plots of the variation of the initial velocity with substrate concentration at (with increasing slope) 0.0, 1.5, 3.0, and 12.0 nM Boc-(d)Val-Leu-(l)boroArg measured for the steady-state phase of p-nitroaniline release. Concentration of OpB was 4.4 nM. B, progress curves for p-nitroaniline release starting with 1 mM substrate and 4 nM OpB and the indicated concentrations of Boc-(d)Val-Leu-(l)boroArg. All kinetics were carried out at 25 °C, 0.1 M Tris buffer, pH 8.0, with 1 mM EDTA.
pSKOpBH652A were digested with NcoI and transformed into E. coli XL1-Blue MRF9. The mutation in plasmids pSKOpBH652Q and pSKOpBH652A was verified by digestion with BglI and EheI restriction enzymes, respectively. The mutations were also confirmed by DNA sequence analysis of the mutated plasmids prepared in E. coli DH5α cells, using a sequencing oligo primer 5’-GGATCCGCAATATTAACGAGTACATGAAA-3’ and the PRISM™ Ready Reaction dideoxy terminator kit from Perkin-Elmer and an Applied Biosystems model 373 DNA sequencer. The sequences obtained showed that the only alterations in the mutated genes were those desired.

The mutated oligopeptidase B was expressed and purified as the wild-type enzyme in E. coli XL1-Blue, except that the enzymes were produced at 30 °C rather than at 37 °C.

Inhibition Studies of OpB by t-butoxycarbonyl-(D)Val-Leu-(L)bora-Arg—The Boc-(D)Val-Leu-(L)boraArg-pinanediol-benzenesulfonate was synthesized according to published methods (25). All kinetic assays were carried out at 25 °C. First, the $K_m$ was determined for $N$-benzoyl-Arg-$p$-nitroanilide-HCl as 0.24 ± 0.06 mM, compared with 0.25 mM reported by Kanatani et al. (23), along with a $k_{cat}$ of 24.5 ± 0.07 s⁻¹ (the number quoted in Ref. 23 is low by a factor of 1000, perhaps the result of an incorrect dilution factor). The Boc-(D)Val-Leu-(L)boraArg-pinanediol-benzenesulfonate (at 0.75 mg/ml) was incubated in 0.1M Tris buffer, pH 8.0, containing 1 mM EDTA, to hydrolyze the pinanediol ester of the boronic acid. A solution of OpB (4.4 nM) was then incubated with the inhibitor solution for 1–2 h at 25 °C, and then the activity was assayed for 3 min. Preincubation of the inhibitor with OpB produced a lag phase for $p$-nitroaniline release before the steady-state kinetic phase was reached. A Lineweaver-Burk plot of the data collected during the steady-state phase indicated the presence of competitive inhibition and a $K_i$ of 3.1 nM (Fig. 1A). Care must be exercised in the kinetic studies since OpB is subject to inhibition at substrate concentrations in excess of 0.5 mM. When the inhibitor and substrate were premixed and the reaction was initiated by the addition of OpB, the progress curves for $p$-nitroaniline release showed that the magnitude of the absorbance plateau varied inversely with inhibitor concentration (Fig. 1B). These experiments strongly imply that the inhibitor is of the “slow-binding” type (25).

RESULTS AND DISCUSSION

Fig. 2 shows spectra of PO between pH 5.14 and 8.22 in the absence and presence of benzoyloxy carbonyl-Pro-Pro-Proline, a potent inhibitor (26) that forms a tetrahedral hemiacetal with a serine at the active center (27). Two resonances are observed under all conditions at 15.8 and 17.0 ppm; they both

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2 Boc-(D)Val-Leu-(L)boraArg-pinanediol-benzenesulfonate is a tripeptide with a $-\text{B(OH)}_2$ group in place of the $\alpha$-COOH at its carboxyl terminus.

3 L. Polgar, unpublished observations.
appear to be pH-independent to pH 8.88 (not shown). Fig. 3 shows spectra at 10 °C for OpB with pH-independent resonances at 16.5 and 17.8 ppm between pH 6.50 and 9.46 that are no longer detectable at pH 9.75. There are additional resonances observed at 13.5 and 14.3 ppm, the latter weaker than the former but quite evident in several spectra, especially at 2 °C (not shown). Fig. 4 shows spectra of OpB (middle), with excess antipain (top, an aldehyde-type inhibitor as is benzoxycarbonyl-Pro-Prolinal for PO), and for the H652A active center variant of OpB (bottom), all at pH 8.1–8.2. The much sharper resonances observed at 14.2 and 13.3 ppm in the presence of the peptide aldehyde inhibitor are not surprising since the inhibitor would tend to make the structure more rigid, perhaps also protecting the histidines in question from exchange with solvent, the principal mechanism responsible for the line width of such resonances. The chemical shifts of the resonances are superimposable within experimental error, as are those observed for the H652Q variant (not shown). One-dimensional difference nuclear Overhauser enhancement spectroscopy (NOESY) experiments (Fig. 5) were carried out on OpB at pH 7.94, showing that the resonance at 17.9 ppm (bottom) correlates with resonances at 13.6, 11 (broad), and 7.8 ppm while the 16.5 ppm resonance (top) correlates with resonances at 11 (broad), 9.6, and 8.4 ppm. It should be noted that the resonances are already broad due to the size of the enzyme, and the high frequency ones are further broadened by exchange (NHs attached to an aromatic imidazolium ring are being reported). Therefore, integration, especially in a difference spectrum, is of virtually no value, and the number of protons represented by each resonance is difficult to ascertain. Fig. 6 shows that addition of Boc-(D)Val-Leu-(L)boroArg-pinanediol-benzenesulfonic acid to OpB produces an additional resonance at 15.8 ppm, whereas the resonances at 17.9 and 16.6 ppm experience shielding to 17.2 and 16.4 ppm, respectively. In no other figures of OpB (Figs. 3, 4, or 5) is the 15.8-ppm resonance visible, hence it can be assigned to the OpB-boronate complex with confidence.

Based on these results, and in comparison with the chemical shifts of nitrogen-bound protons at the active center His\(^{\text{N}}\) of chymotrypsin, trypsin, and at His\(^{\text{64}}\) of subtilisin (represented in Scheme I), we interpret the results as follows. (a) The resonance pertinent to the active center His N\(^{\text{H}}\) is not visible in the spectra of wild-type OpB and PO between pH 5.1 and 9.5. Such resonances have been detected by several groups both for uncomplexed and complexed chymotrypsin, trypsin, and subtilisin (8–18). (b) There are two new resonances found in PO and OpB, as well as in the active center H652A variant of OpB, whose chemical shifts are virtually pH-independent up to a pH of 8.8 in PO and to 9.5 in OpB. The resonances are in very slow exchange with any other in their environments. The two resonances therefore do not pertain to the active center His. The chemical shifts of the two resonances (between 15.8 and 18 ppm), however, are appropriate to His in its protonated state (see Scheme I and Refs. 8–18) and further imply that the protons represented by the resonances are strongly hydrogen...
bonded to some hydrogen bond acceptor. (c) The two resonances observed in PO and OpB persist in the presence of aldehyde-based transition-state analogs, and there are no additional resonances observed in the presence of these inhibitors (anti-pain for OpB and benzylxoycarbonyl-Pro-Pro-Prolinal for PO). (d) The difference in chemical shift between the two resonances observed in both wild-type and variant enzymes remains the same under all conditions examined (pH, added inhibitor) and is virtually the same for PO and OpB. (e) The observation of the same resonances in the H652A (and H652Q) variant of OpB strongly suggests that this variant is correctly refolded as the presence of these resonances is clear evidence for the formation of the proper tertiary structure. At the same time, the variant enzyme is completely inactive (activity 0.01%), which confirms that the active site His has indeed been altered. (f) Nuclear Overhauser enhancement experiments provide strong evidence that the two resonances observed in OpB pertain to different histidines. We suggest that the 17.9- and 16.6-ppm resonances represent the N\textsuperscript{1H} of two different histidines, whereas the NOEs imply that the resonance at 13.5 may correspond to the N\textsuperscript{2H} of the histidine with N\textsuperscript{1H} at 17.9 ppm. This resonance at 13.5 ppm is visible in several spectra, as is a different one at 14.2 ppm (see Figs. 3 and 4, and especially Fig. 4, top spectrum, in the presence of antipain that apparently slows down the exchange rate of these two protons). The resonance at 14.2 ppm likely corresponds to an N\textsuperscript{2H} residing on the histidine with an N\textsuperscript{1H} at 16.6 ppm. The resonances at lower frequency between 9.6 and 7.8 ppm could pertain to the C2 and C4 hydrogens of the imidazole rings and probably to some backbone NHs. These assignments are in accord with a recent study by Markley and Westler (12) on chymotrypsinogen and affirm that the two histidines are in their histidinium ionization state. (g) pH titration of the solution of OpB shows the p\textsubscript{Ka} of the two histidines to be greater than 9.5, and those of PO to be greater than 8.9. For comparison, the p\textsubscript{Ka} for His\textsuperscript{57} in uncomplexed chymotrypsin is 7, and the chemical shift of the active center His\textsuperscript{57} varies with pH (8–10, 14). (h) The peptide boronic acid Boc-(D)Val-Leu-(L)boroArg turned out to be a very potent competitive slow binding inhibitor (K\textsubscript{i} \sim 3 \text{mM}) of OpB, in whose presence the spectrum of OpB exhibits a single new resonance at 15.8 ppm. In the chymotrypsin/trypsin and subtilisin class, the serine-bound boronates exhibit two resonances pertinent to N\textsuperscript{1H} and N\textsuperscript{2H} (14). The observation of only a single resonance with a chemical shift of 15.8 ppm pertinent to the complex is not only appropriate for a nitrogen-bound proton at histidine in complexes of serine proteases with peptide bo-
ronic acids but the behavior is also consistent with the boron being bound to a His at N^2 (in this case His^652), or to Ser and His concurrently, rather than to a serine at the active center (14, 28) and the resonance being pertinent to N^1His side rather than to a serine in the active center. While both non-catalytic histidines rather than the one at the active center. Further research will be required to determine which two of the five remaining conserved histidines are being observed in these experiments and are deduced to participate in strong hydrogen bonds.

The inability to observe the active center His resonances in spectra of OpB and PO strongly suggests that the Asp-His hydrogen bond is much weaker in these enzymes than in the chymotrypsin/subtilisin classes of serine peptidases. The absence of such a strong hydrogen bond between the active center Asp and His in OpB and PO suggests that: (a) there is no LBHB at this position in these two enzymes in the uncomplexed form or in the tetrahedral complexes formed with the aldehyde-based so-called “transition-state” analogs; (b) there is an LBHB in the tetrahedral complex formed with the peptide boronic acid, which installs a negative charge on the N^2 side as well.

One may also conclude (as we did in Ref. 13) that near neutral pH values, the negative charge at the N^2 side creates a better transition-state Analog complexes in terms of electrostatic interactions (AspCOO^- HisH^+ SerX^-) than the aldehyde, which appears to form a neutral tetrahedral complex (AspCOO^- HisH^+ SerX); compare results on OpB with antipain and the peptide boronic acid and the results on trypsin in the presence of leupeptin in Ref. 16. In the case of OpB, the boronate complex with the tetrahedral anionic character evidently induces a stronger hydrogen bond between the Asp and N^1Hi. These results are consistent with previous reports on transition-state analogs that install a negative charge on the N^2 side, such as complexes of peptide boronates (13–16, 28), peptidyltrifluoromethylketones (29–30), as well as the monoiso-proplyphosphoryl Ser^195 derivatives of chymotrypsin, trypsin, and subtilisin (31), all of which uniformly raise the pK_a (increase the basicity) of the active center histidine substantially. Similarly elevated pK_a of the active center His in going to the transition state would be expected with the developing oxanion, thereby reducing the ΔpK_a between the histidine and the serine, and with possible concomitant strengthening of the hydrogen bond between the Asp and N^1Hi.

The results also confirm that such exceptionally strong hydrogen bonds, reflected by such unusually large chemical shifts, will be revealed in the structures of many enzymes. Additional examples are provided by some coenzyme-dependent enzymes: the pyridoxal phosphate-dependent enzymes that gave rise to several resonances with chemical shifts between 13–20 ppm, one of them assigned to the pyridinium N1-proton (32–35); and the thiamin diphosphate-dependent pyruvate de-carboxylase that exhibits a chemical shift of 17.2 ppm, a resonance as yet unassigned. In summary, there are distinct differences found between the active centers of this new prolyl oligopeptidase class of serine peptidases and the well studied chymotrypsin/trypsin and subtilisin classes. (a) There is no LBHB in the ground state, but there are two non-catalytic histidines that appear to participate in LBHBs in the prolyl oligopeptidase class. (b) One can design potent, slow-binding peptide boronic acid-type inhibitors for both the prolyl oligopeptidase, and the chymotrypsin/trypsin and subtilisin classes. But, while the former appears to form a boronate complex to His N^2 (or simultaneously to His and Ser, a possibility that is difficult to differentiate by NMR methods) at the active center, the chymotrypsin/trypsin and subtilisin classes form a serine-bound boronate. It is concluded, therefore, that there are subtle novel features observed in both the ground state and in the transition-state type complexes in this prolyl oligopeptidase class of serine peptidases that have not been previously seen in any other serine proteases.

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4 D. Bao and F. Jordan, unpublished data.

5 D. Zhang, F. Guo, and F. Jordan, unpublished observations.
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