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Multinuclear Magnetic Resonance Studies on Serine Protease Transition State Analogues

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^{31}P Nuclear Magnetic Resonance (NMR) studies were performed on mono- and diisopropylphosphoryl derivatives of α-chymotrypsin, trypsin, and subtilisin. Questions addressed included the pK_a of the active center Asp...His...Ser triad in both species. While the pK_a in the diisopropylphosphoryl derivatives is near 7.4 (found in this and other laboratories earlier) and reflects a nearly normal imidazolium titration curve, the apparent pK_a in the monoisopropylphosphoryl enzymes (obtained by "aging" of the diisopropylphosphoryl derivatives and monitored by ^{31}P NMR) is between 9.7 and 11.4 depending on the protease. This latter "titration" of the ^{31}P NMR signal is reversible and presumably reflects the interaction of the imidazolium positive charge with the monoanionic phosphodiester. Of the two tetrahedral intermediates, the properties of the monoisopropylphosphoryl enzyme are probably more representative of the tetrahedral oxyanionic intermediate invoked during peptide hydrolysis. The same NMR technique was used to determine the action of PAM (pyridine-2-aldoxime methiodide, a known "antidote" for acetylcholinesterase inactivated by diisopropylfluorophosphate), on the inactivated enzymes. It was clear that the "antidote" could reverse the diisopropylphosphorylation but was ineffective on the monoisopropylphosphoryl ("aged") enzyme.

^{11}B NMR studies were performed on phenylboronic (PBA) acid and 3,5-bis-trifluoromethylphenylboronic acid in the absence and presence of chymotrypsin and subtilisin. At 22°C the former, but not the latter, compound was in fast exchange between the free and enzyme bound states. The relaxation parameters could be calculated for the bound PBA in chymotrypsin and the fluorinated analogue in subtilisin and clearly indicated that the boron nucleus was tetrahedral in the active centers, a good analogue for the tetrahedral oxyanionic intermediate.

Key words: transition state analogues, MIP-subtilisin, phenylboronic acid, bistrifluoromethylphenylboronic acid, MIP-chymotrypsin, MIP-trypsin, DIP-subtilisin, DIP-chymotrypsin, DIP-trypsin, ^{11}B nuclear magnetic resonance, ^{31}P nuclear magnetic resonance

Nuclear Magnetic Resonance (NMR) studies on serine proteases have provided detailed insight about the active center in solution not available from any other technique. Perhaps some of the best information is available about the active center His from a variety of NMR approaches: ^{1}H on the hydrogen bonding proton between Asp and His

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[1–6], ^1H on the aromatic His C2-H [7–10], and ^{15}N [6,11] and ^{13}C [12,13] on specifically enriched samples of the bacterial α-lytic protease. With the successful early applications of transition state analogues as enzyme inhibitors [14,15], it became apparent that the tetrahedral intermediates covalently bonded to the active center Ser may bear resemblance to the tetrahedral oxyanionic intermediate (and perhaps preceding transition state) invoked in the hydrolytic pathway of peptide bonds. We here report studies of two classes of such putative transition state analogues: (1) a tetrahedral neutral phosphotriester, the diisopropylphosphoryl derivatives (DIP) produced by reaction with diisopropylfluorophophate (DIFP); a tetrahedral monoanionic phosphodiester, the monoisopropylphosphoryl derivatives (MIP) produced from enzyme catalyzed hydrolysis of the DIP analogues, both readily discerned by ^{31}P NMR since they have different chemical shifts and pH-dependent behavior; and (2) the complexes formed with boronic acids that, according to x-ray evidence in the solid state [16–18], form tetrahedral monoanionic complexes with the active center serine.

We have employed these tools in several hitherto unexploited ways. The ^{31}P NMR of MIP and DIP subtilisin, chymotrypsin, and trypsin was monitored to determine the apparent pK near the ^{31}P nucleus and the pH dependence and mechanism of reactivation of the DIFP-inactivated enzyme with the well-known antidote pyridine-2-aldoxime methiodide (PAM).

^{11}B NMR was useful in allowing us to determine the state of hybridization around the boron atom when enzyme-bound and as to whether or not the boronic acid was bound in a single or at multiple binding sites.

MATERIALS AND METHODS

Enzyme Assays and Inactivation

Subtilisin Carlsberg and trypsin were purchased from Sigma (St. Louis) and α-chymotrypsin from Worthington (Freehold, NJ). Chymotrypsin and trypsin were assayed according to Hummel [20] and subtilisin according to Jordan et al. [10]. Modification with DIFP was performed according to Jordan et al. [10]. DIFP was purchased from Aldrich (Milwaukee, WI) and PAM from Sigma. All buffer components and inorganic compounds were the highest purity available.

Nuclear Magnetic Resonance Studies

All NMR studies were performed on an IBM WP-200 SY spectrometer equipped with a thermostatted probe. ^{31}P was observed at 81.026 MHz in a 10 mm O.D. glass tube, spectra were referenced against external 85% H_3PO_4. ^{11}B was observed at 64.2 MHz employing a probe specially constructed for this nucleus and accommodating a 10 mm O.D. quartz (Wilmad) tube, and referencing against external $B(OCH_3)_3$. It is noteworthy that the normal broad band probes and normal glass tubes were not usable for these experiments at the concentration of boronic acids employed since the glass in both the probe and the tube would give rise to a very broad background in the chemical shift range of interest. By contrast, with the help of the dedicated ^{11}B probe and special quartz tubes, concentrations of inhibitors at 1 mM and even lower were readily observed and quantified. The quadrupolar nature of ^{11}B ($I = 3/2$) offers some important advantages for S/N enhancement, the very short relaxation time enabling use of recycle times of 0.1 sec and even less.

RESULTS

^{31}P NMR on DIP and MIP Enzymes

On the conversion of DIP to MIP derivatives. A typical ^{31}P NMR spectrum of a mixture of DIP and MIP enzymes is illustrated in Figure 1. Similar data have been presented by Gorenstein on DIP chymotrypsin [21], Markley on DIP-trypsin and DIP-chymotrypsin [22], Van der Drift [23,24] on DIP-chymotrypsin and DIP-subtilisin, and Jordan et al. [10] on DIP-subtilisins. As a function of pH the DIP derivative is converted to the MIP derivative as reported by Van der Drift [23,24]. Most importantly, the DIP and MIP derivatives give rise to ^{31}P chemical shifts that are readily differentiated even at 81 MHz.

Identification of the species reacting with PAM. PAM (pyridine-2-aldoxime methiodide) is a known antidote for the inactivation of acetylcholinesterase with DIFP [25]. ^{31}P NMR offers an excellent tool with which to address the question as to whether or not the DIP or MIP derivative reacted with PAM during the reactivation, and to monitor the pH dependence of this reactivation. For purposes of this experiment the DIP derivative was partially aged so that nearly equal concentrations of MIP and DIP derivatives were present. Upon addition of PAM (Fig. 2), the amount of DIP present clearly diminished, while the concentration of MIP derivative remained unchanged. A new ^{31}P resonance with a pH-independent chemical shift appropriate to an aliphatic dialkyl phosphate (in this case diisopropylphosphate) appeared concomitant with the disappearance of the resonance corresponding to the DIP derivative. Clearly, PAM reacted only with the DIP derivative. The rate of reactivation was studied at pH 7.8, 7.4, and 6.4. Reactivation proceeded optimally at around pH 7.0 (Fig. 3). To confirm the NMR results, the rate of reactivation with PAM was also determined by a kinetic assay. Three solutions containing identical concentrations of totally inactive DIP-subtilisin and PAM were incubated at 30°C at pH 7.9, 7.3, and 6.3, respectively, for 12 hours. The activity at pH 7.9 was ca. 35% lower, and at pH 6.3 ca. 15% lower than at pH 7.3.

pH dependence of the ^{31}P chemical shift in DIP and MIP enzymes. The chemical shift of the phosphorus resonance was recorded over a wide pH range for MIP and DIP derivatives of subtilisin, trypsin, and chymotrypsin. The titration curves were reversible as demonstrated by the fact that the experimentally determined chemical shifts were identical at the same pH when titrating either to higher or to lower pH. The pK's deduced from the titration curves are listed in Table I. The curves themselves are presented in Figures 4–6.

^{11}B NMR on Phenylboronic and 3.5-bis-Trifluoromethylphenylboronic Acids in the Presence of Chymotrypsin and Subtilisin

In a preliminary communication it was reported from this laboratory that under fast exchange conditions between the free and enzyme-bound boron nucleus, the chemical shift and the relaxation parameters pertinent to the enzyme bound species can be deduced [19]. The significance of the latter quantities is that they enable estimation of the quadrupolar coupling constant and from that the symmetry of the boron at the active center, i.e., whether it is trigonal or tetrahedral.

While the preliminary results were obtained on PBA attached to chymotrypsin, such studies have now been extended to subtilisin and employed a second boronic acid analogue.

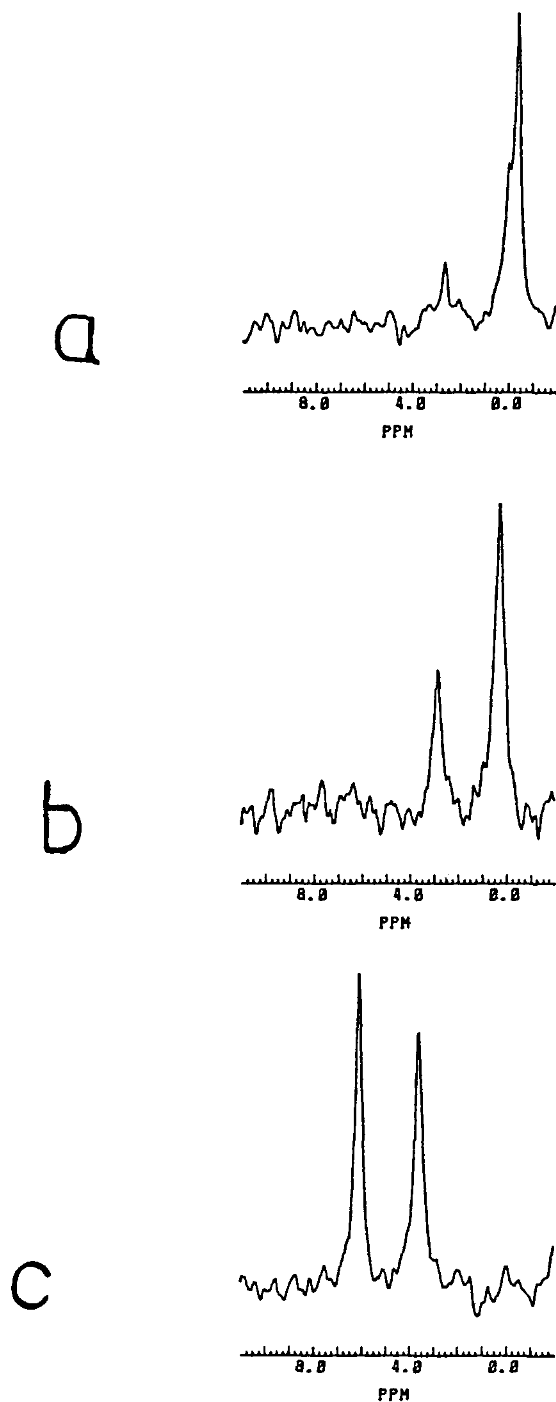

Fig. 1. ^{31}P NMR spectrum of a mixture of MIP and DIP complexes (i.e., a partially aged sample) of trypsin (**a**), α-chymotrypsin (**b**), and subtilisin (**c**) in 0.01 M Tris buffer pH 7.8. The lower field peak in each spectrum is due to the MIP-enzyme.

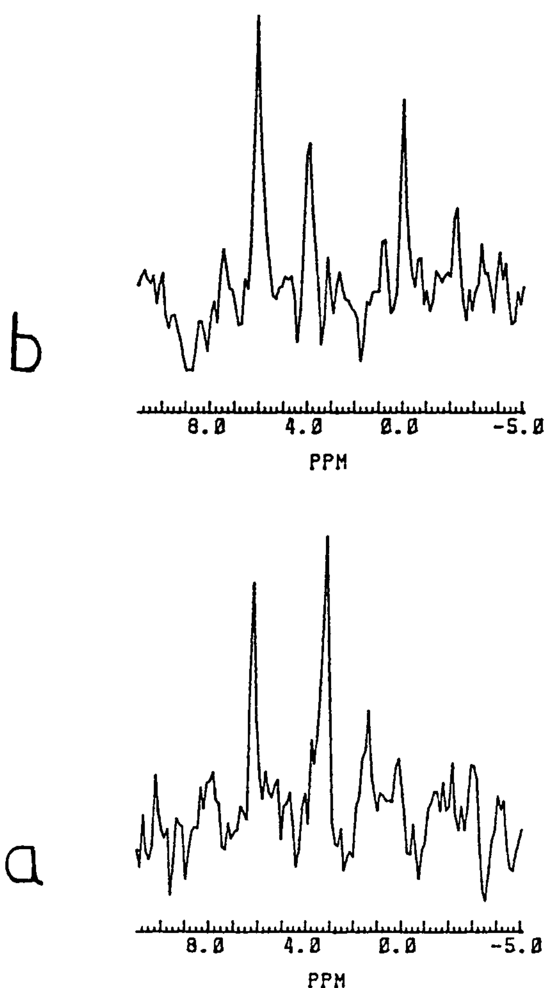

Fig. 2. ^{31}P NMR spectrum of a partially aged mixture of MIP and DIP subtilisins (1.8 mM total inhibited enzyme) before (a) and 14 h after (b) the addition of 0.13 M PAM, pH 7.4.

The ^{11}B resonance of 2.9 mM 3,5-bis-trifluoromethylphenylboronic acid (BTFPBA) at pH 4.0 when observed in the presence of 0.2 mM chymotrypsin at 22°C underwent no change in chemical shift but broadened with increasing temperature (Fig. 7) indicating a system in slow exchange. Raising the temperature to 36°C shifted the system into the fast exchange regime and enabled us to perform a titration of 2.91 mM BTFPBA with limiting concentration (0.012–0.12 mM) of chymotrypsin (see Fig. 8). The control experiment in which the BTFPBA resonance was titrated with increasing concentrations of DIP-chymotrypsin led to both upfield chemical shift (about 60% of the amount achieved by the same concentration of active enzyme) and increased linewidth (Fig. 8).

The interaction of BTFPBA with subtilisin at 22°C and pH 4.2 reflects a system under fast exchange (data not shown). The titration of 2.91 mM BTFPBA with increasing concentrations of subtilisin (0.019–0.15 mM) at this pH shifted the ^{11}B

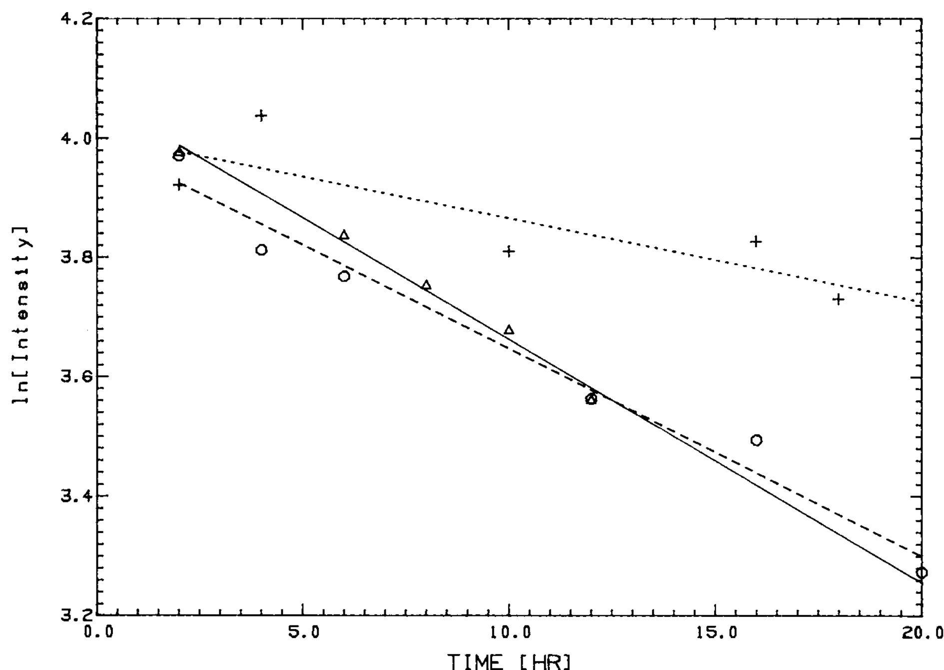

Fig. 3. ^{31}P NMR results on the reactivation of DIP-subtilisin at pH 7.8 (+), 7.4 (△), and 6.4 (○) with 0.13 M PAM.

resonance to higher field. The resonance also exhibited excess linewidth compared to a similar titration curve performed with DIP-subtilisin. The latter control titration led to no change in the ^{11}B chemical shift, suggesting no binding to DIP-subtilisin by BTFPBA. An analysis of the chemical shift and relaxation data according to the protocol outlined before [19] gave the following parameters for the bound B atom: the chemical shift is −12.3 ppm, the quadrupolar coupling constant is 1.0 MHz, and the $K_{dissociation}$ is 0.1 mM. The chemical shift and the quadrupolar coupling constant of the bound B nucleus are totally consistent with a tetrahedral environment [19].

DISCUSSION

The pK_a of the Active Center in MIP and DIP Complexes

The most striking feature of the results in Table 1 concerning the pH-dependent behavior of the ^{31}P resonance is the vast difference in apparent pK's between the MIP and DIP analogues. While the DIP analogues have pK's near 7.3–7.4 [10,21–24], the

TABLE I. pK_a's in Monoisopropylphosphoryl (MIP) and Diisopropylphosphoryl (DIP) Enzymes at 22°C

	pK_a	
Enzyme	DIP	MIP
Subtilisin	7.4	11.4
α-Chymotrypsin	7.3	10.3
Trypsin	7.3	9.7

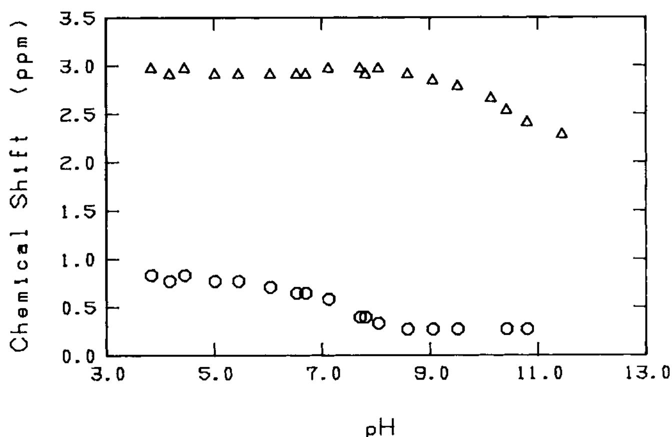

Fig. 4. pH dependence of the ^{31}P NMR chemical shifts in the MIP (△) and DIP-α-chymotrypsin (○).

MIP derivatives have consistently higher apparent pK's between 9.7 and 11.4, depending on the enzyme examined. Given that phosphorylation had been proven to take place at the active center, and since the phosphotriester in the DIP derivative has no ionizable groups, whereas the phosphodiester in the MIP derivative can be expected to have a pK below 2, the apparent pK's can be assumed to pertain to the ionization of the active center His according to Figure 9 below. The pK values observed in the DIP derivatives (very similar to the pK of the active center His in the native enzymes) give particularly strong support for this assumption. The reversibility of the titration curves also supports such an assumption, since pH-dependent denaturation is unlikely to be reversible. The remarkable increase in the basicity of the active center His in the MIP derivative can probably be attributed to the presence of a negative charge on the MIP that can stabilize the imidazolium of the His, which now experiences stabilization from both sides, i.e., also from the Asp carboxylate at the active center. In an earlier contribution we had demonstrated that the hydrogen bonded proton between Asp and His is stabilized by the

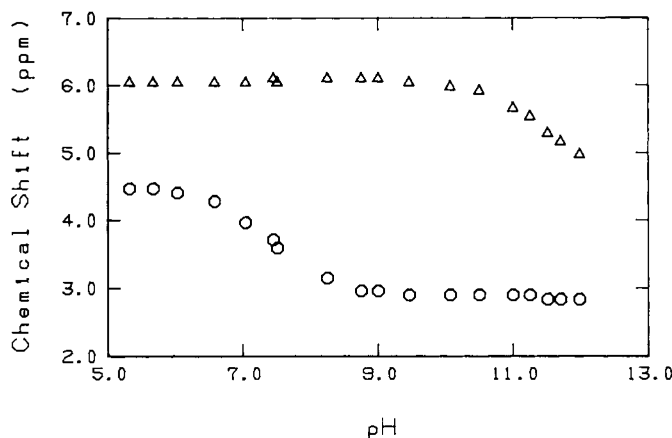

Fig. 5. pH dependence of the ^{31}P NMR chemical shifts in the MIP (△) and DIP-subtilisin (○).

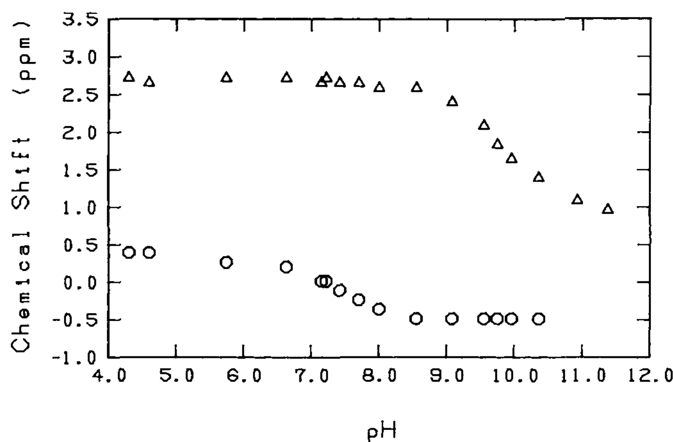

Fig. 6. pH dependence of the ^{31}P NMR chemical shifts in the MIP (△) and DIP-trypsins (○).

presence of a negative charge on the other side of the $HisH^+$ residue attached either covalently or noncovalently to Ser [5]. The results here reported are complementary to those earlier ones and suggest that the presence of the negatively charged tetrahedral adduct on the Ser can increase the pK of the active center His by 2.5–4 units depending on the enzyme. On account of its charge, the MIP derivative is a better analogue of the tetrahedral oxyanionic intermediate than the DIP derivative. The results suggest that one can argue for a stabilization of the $HisH^+$ by the MIP^- by approximately 3.5–5.5 Kcal/mol (corresponding to the 2.5–4 unit pK shift observed). The magnitude of this stabilization is probably similar to that experienced by the mutual stabilization between the $HisH^+$ and the developing oxyanionic intermediate (and preceding transition state) derived from peptide substrates. Oligonucleotide directed site-specific mutagenesis of one of two oxyanion hole residues Asn-155 to Leu-155 of *Subtilisin amyloliquefaciens* led to no change in the K_M but a 200–300-fold diminution in the k_{cat} toward a peptide substrate when compared to the wild-type enzyme [26]. The activation energy lowering that gives rise to this rate acceleration (attributed to just one of the two residues responsible for the "oxyanion hole"), if doubled, is in order of magnitude agreement with the energy changes suggested by the NMR results on the MIP-proteases for the pK shift on the catalytic His imidazole going from the ground state to the oxyanionic intermediate. The results here reported refer to an intermediate. But clearly, the transition state preceding this intermediate would benefit from the same type of stabilization claimed above, albeit of perhaps smaller magnitude. Therefore, a decrease in activation energy not exceeding 3.5–5.5 kcal/mole in trypsin, chymotrypsin, and subtilisin may result from the mutual charge stabilization between $HisH^+$ and the oxyanionic intermediate, at least some of the energy being derived from interactions with the oxyanion hole. These pK's observed in the MIP derivatives should be more similar to those expected for the His in the transition states than those observed in the ground state, and provide the first estimates for such pK shifts during the reaction. It is noteworthy, that the ^{1}H NMR experiments reported for the boronate complexes of chymotrypsin and subtilisin [1–5] indicated no change in the pK of the active center His between pH 5–9, and, based on the chemical shift of the now famous Robillard-Shulman resonance, suggested a $HisH^+$ ionization state, hence a substantial pK shift consistent with the present results. From the

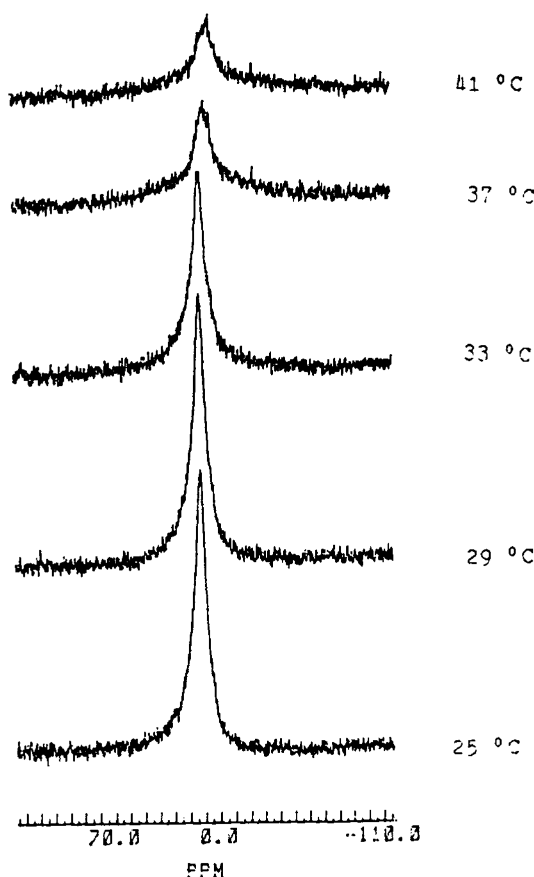

Fig. 7. Temperature dependence of the ^{11}B NMR resonance of a 2.9 mM solution of BTFPBA in the presence of 0.2 mM α-chymotrypsin at pH 4.0.

point of view of the accepted mechanism for serine proteases, the enhanced basicity of His in the oxyanionic intermediate will assure the retention of the proton on $HisH^+$ through this step and its availability for the subsequent transfer to the amide leaving group.

Experiments on the Reaction of DIP Derivatives With PAM

^{31}P NMR is a powerful tool with which to study the formation of the DIP and MIP derivatives. No extraneous ^{31}P resonances were observed during the reaction, hence there were no complicating side reactions or accumulating intermediates. The addition of PAM led to the formation of an acyclic phosphodiester from the DIP derivative only. The antidote therefore is only effective before the process of "aging" sets in. The approximate pH dependence of the action of PAM suggests that the reaction is modulated by two pH-dependent groups leading to a pH optimum around 7: an enzymic base with a pK near 7 and the oximate anion (pK = 7.6). A likely mechanism for this process is outlined in Figure 10.

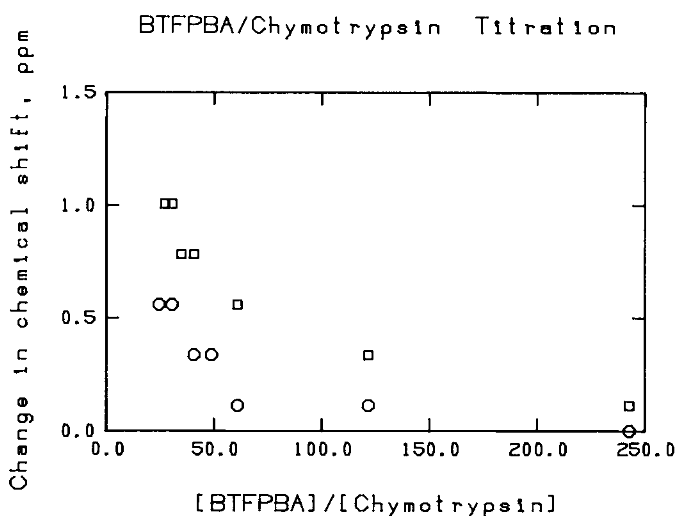

Fig. 8. Change in ^{11}B chemical shift of a 2.9 mM solution of BTFPBA upon the addition of α-chymotrypsin (○), and DIP-chymotrypsin (□) at 36°C and pH 4.0.

^{11}B NMR Results

Beyond the example we had reported in a preliminary communication on the binding of PBA to chymotrypsin [19], we now have data on several other systems from which some general conclusions can be drawn concerning the technique. The most important limitation of the technique is that the relaxation data concerning the enzyme-bound B atom can be obtained in solution only for a system in which the B atom is in fast exchange between the free and the enzyme-bound forms. The magnitude of the quadrupolar coupling constant [27] deduced for the enzyme-bound B atom indicates a

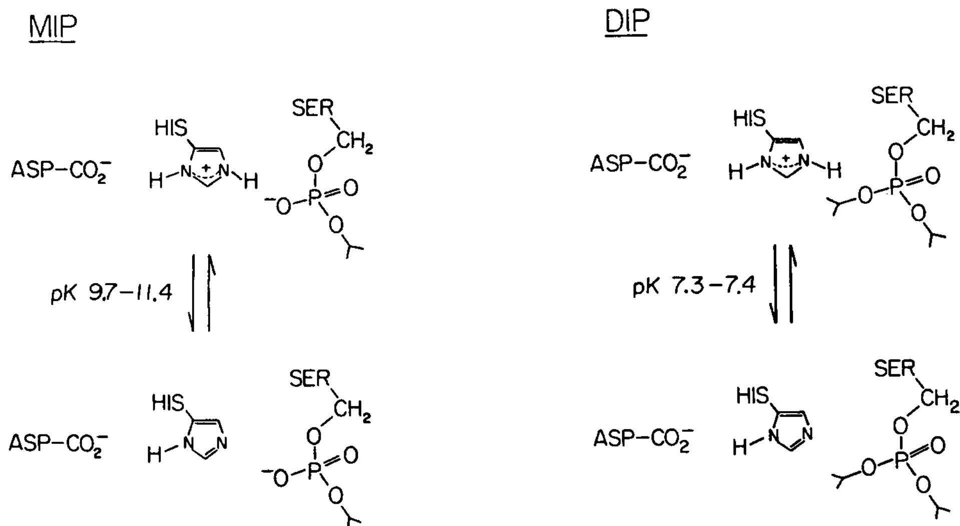

Fig. 9. Ionization of the active center His in DIP and MIP-proteases.

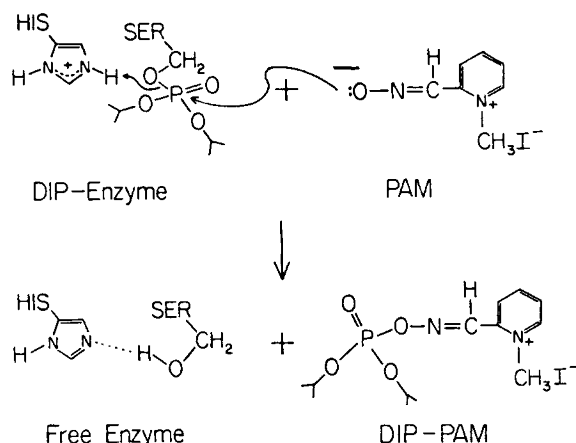

Fig. 10. Likely mechanism for reaction of DIP derivatives with PAM.

tetrahedral environment for PBA attached to chymotrypsin. BTFPBA binds to chymotrypsin more strongly than does PBA and at pH 4.0 and 22°C is in slow exchange, but at 36°C shifts into the fast exchange regime. The "binding" curves (i.e., changes in the ^{11}B chemical shift with addition of increments of active chymotrypsin or DIP-chymotrypsin) presented in Figure 8 demonstrate the binding of BTFPBA to both native and DIP-chymotrypsin. These observations, taken together with the previous observation that PBA is not complexed to DIP-chymotrypsin [19], and that BTFPBA does not bind to DIP-subtilisin indicate more than one possible binding site for BTFPBA at pH 4.0 on native chymotrypsin. The possibility that under these conditions boronic acids bind to chymotrypsin in two different modes was raised by the x-ray results of Tulinsky and Blevins [17]. The crystals used in that study were grown under acidic conditions, and the structure of chymotrypsin was solved as a dimer, in which the phenylethylboronic acid was bound in a tetrahedral complex at one active center Ser, but in a noncovalent mode near the second active center. It is also clear, however, that BTFPBA does bind to subtilisin at a single site at pH 4.2. In order to draw conclusions about the chemical shift of the bound boron atom, the unbound boron should be predominantly trigonal, otherwise the difference between the free and bound chemical shift of a tetrahedral boron may be too small to quantify. The ^{11}B resonance is already rather broad owing to the quadrupole, and the chemical shift cannot be measured better than ± 0.1 ppm. These factors limit the pH range in which BTFPBA may be employed since it undergoes trigonal to tetrahedral transition at pH 7.0.

Our experiments have not addressed the question of whether or not the boron atom of the inhibitor is bound to the catalytic Ser or His. X-ray crystallographic results uniformly suggested that the boronic acid inhibitors were bound to the catalytic Ser. A very recent ^{15}N NMR study on α-lytic protease specifically labeled at the two imidazole nitrogens of the single His at the active center suggested that weak, nonspecific inhibitors such as PBA are bound to the His, while the stronger peptide-based boronic acids are bound to the Ser [28]. The ^{11}B NMR parameters deduced for the enzyme-bound boron atom do not provide guidance on this point. In a number of model studies we observed that the chemical shift of ^{11}B whether bound to a nitrogen or oxygen ligand will fall in the

same general range for tetrahedral species, i.e., one cannot differentiate the two types of ligands based on chemical shift alone. We also observed, however, that ^{14}N (the most abundant nitrogen isotope that is itself quadrupolar) induces a significantly large line broadening (relaxation) in the ^{11}B resonance. This effect will prove useful in assigning the type of ligand bonded to boron at least in model systems, and perhaps on enzymes as well.

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REFERENCES

1. Robillard G, Shulman RG: J Mol Biol 71:507, 1972.
2. Robillard G, Shulman RG: J Mol Biol 86:519, 1974.
3. Robillard G, Shulman RG: J Mol Biol 86:541, 1974.
4. Markley JL: Biochemistry 17:4648, 1978.
5. Jordan F, Polgar L: Biochemistry 20:6366, 1981.
6. Bachovchin WW: Proc Natl Acad Sci USA 82:7948, 1985.
7. Markley JL, Porubcan MA: J Mol Biol 102:487, 1976.
8. Markley JL, Ibanez IB: Biochemistry 17:4627, 1978.
9. Omar S, Brown MF, Silver D, Schleich T: Biochim Biophys Acta 578:261, 1979.
10. Jordan F, Polgar L, Tous G: Biochemistry 24:7711, 1985.
11. Bachovchin WW, Roberts JD: J Am Chem Soc 100:8041, 1978.
12. Hunkapiller MW, Smallcombe SH, Whitaker DR, Richards JH: Biochemistry 12:4372, 1973.
13. Bachovchin WW, Kaiser R, Richards JH, Roberts JD: Proc Natl Acad Sci USA 78:7323, 1981.
14. Wolfenden R: Acc Chem Res 5:10, 1972.
15. Lienhard GE: Science 180:149, 1973.
16. Matthews DA, Alden RA, Birktoft JJ, Freer ST, Kraut J: J Biol Chem 250:7120, 1975.
17. Tulinsky A, Blevins RA: J Biol Chem 262:7737, 1987.
18. Bone R, Shenvi AB, Kettner CA, Agard DA: Biochemistry 26:7609, 1987.
19. Adebodun F, Jordan F: J Am Chem Soc 110:309, 1988.
20. Hummel BCW: Can J Biochem Physiol 37:1393, 1959.
21. Gorenstein DG, Findlay JB: Biochem Biophys Res Commun 72:640, 1976.
22. Porubcan MA, Westler WA, Ibanez IB, Markley JL: Biochemistry 18:4108, 1979.
23. Van der Drift ACM: Doctoral Thesis Utrecht, The Netherlands 1983.
24. Van der Drift ACM, Beck HC, Dekker WH, Hulst AG, Wils ERJ: Biochemistry 24:6894, 1985.
25. Wilson IB, Ginsburg S: Biochim Biophys Acta 18:168, 1955.
26. Bryan P, Pantoliano MW, Quill SG, Hsiao H-Y, Poulos T: Proc Natl Acad Sci USA 83:3743, 1986.
27. Forsen S, Lindman B: Methods Biochem Anal 27:322, 1981.
28. Bachovchin WW, Wong WYL, Farr-Jones S, Shenvi AB, Kettner CA: Biochemistry 27:7689, 1988.