Ionizable P1 Residues in Serine Proteinase Inhibitors Undergo Large pK Shifts on Complex Formation*

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The burial of charged residues in proteins is rare as it is thermodynamically strongly disfavored. However, in “standard mechanism” protein inhibitors of serine proteinases, the P1 residue, which is highly exposed, becomes buried in the S1 specificity pocket of the enzyme. In many enzymes, such as Streptomyces griseus proteinase B (SGPB) the S1 pocket is hydrophobic. We measured the pH dependence of the association equilibrium constant for the interaction of SGPB with turkey ovomucoid third domain P1 mutants, Glu18 OMTKY3 and His18 OMTKY3. In order to eliminate the effects of other ionizable groups on the enzyme and the inhibitor, we divided these pH dependences by the pH dependence of the association equilibrium constant for the Gin18 OMTKY3 mutant. This yielded for Glu18, pK (free inhibitor) of 4.46 ± 0.05 and pK (complex) of 8.74 ± 0.06. For His18 the values are pK, 6.63 ± 0.08 and pK, 4.31 ± 0.07. At low pH values Glu18 variant is a relatively good inhibitor for SGPB. This may be biologically relevant.

Transferring a charge from a high dielectric medium, such as water, to a low dielectric medium, such as organic solvents or interiors of proteins, is not thermodynamically favored (1). Therefore, ionizable side chains buried in proteins should experience large pK shifts. Such shifts are occasionally seen (2–5), but they are rare. While ionizable residues are fairly often buried in their uncharged form, acquisition of charge is most often associated with a conformational change (3, 4) or with dissociation of the ligand-receptor complex. We describe two cases where the complexes persist with the charged form of a residue (6). While the interaction of P1 with the enzyme is energetically the most important, about a dozen of the inhibitors’ residues interact both by main chain-main chain hydrogen bonds and by specific side chain-side chain interactions. Collectively, the interactions of all the contact residues, other than P1, are energetically dominant. They ensure that the same peptide bond in the inhibitory domain acts as the reactive site for numerous different cognate enzymes (10). More importantly, they ensure that, in inhibitor mutants, substitutions of the P1 residue do not shift the reactive site peptide bond and that the mutant P1 side chain is still imbedded in the S1 cavity of the enzyme, even if that interaction alone is energetically adverse rather than favorable (11).

Turkey ovomucoid third domain, OMTKY3, is a widely studied “standard mechanism,” canonical inhibitor of serine proteinases. With its P1 Leu residue, it is a powerful inhibitor of many chymotrypsins, elastases, subtilisins, and two of the many Pronase components, Streptomyces griseus proteinase A (SGPA) and proteinase B (SGPB) (10). All of these enzymes have predominantly hydrophobic S1 pockets and prefer substrates and inhibitors with hydrophobic P1 residues. We have recently acquired 25 different P1 variants (all 20 coded and 5 noncoded) of X18 OMTKY3 and measured their association constants, K, at pH 8.3 with six enzymes, all of which are members of the set listed above (18, 19). In a parallel study, Huang and J ames (12) undertook to obtain high resolution three-dimensional structures of all 20 coded X18 OMTKY3 variants with SGPB. Many of these structures, which crystallized near pH 6.5, especially Glu18 OMTKY3, are already in hand (13). The binding of Glu18 to the S1 specificity pocket of SGPB is similar to the binding of Leu10 (14, 15). The interpretation of the K, (at pH 8.3) and the x-ray data (at pH 6.5) requires knowing whether the Glu18 residue is protonated (Glu-) or deprotonated (Glu-) in complex at these values. When appraised of our results that the pH 6.5 structure was of the protonated form, Glu (16), Huang and J ames (17) raised the pH of their crystals to 10.7. The global structure of the complex was unaltered, but clear evidence was obtained for Glu- rather than Glu (18).

EXPERIMENTAL PROCEDURES

Proteinase—S. griseus protease B, SGBP, was purified from Pronase (Boehringer Mannheim) by the procedure of Jurasek et al. (20) modified in this laboratory (21). The amino acid composition of the isolated enzyme is consistent with the published sequence.

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Expression of Third Domain Variants—The ovomucoid third domain variants are routinely expressed in our laboratory in the periplasmic space of Escherichia coli as fusion proteins with two Z domains of protein A. The fusion protein is purified by affinity chromatography on IgG-Sepharose (Pharmacia Biotech Inc.), which specifically binds the protein A. The fusion protein is purified by affinity chromatography on IgG-Sepharose (Pharmacia Biotech Inc.), which specifically binds the protein A. The released domain is purified by size exclusion and ion exchange chromatography. It is extensively characterized by amino acid analysis, high resolution mass spectrometry, sequencing beyond exchange chromatography. It is extensively characterized by amino acid analysis, high resolution mass spectrometry, sequencing beyond exchange chromatography. It is extensively characterized by amino acid analysis, high resolution mass spectrometry, sequencing beyond exchange chromatography.

RESULTS AND DISCUSSION

The pH dependence of log $K_f$ for three ovomucoid third domain variants, namely ionizable Glu$^{18}$ OMTKY3 and His$^{18}$ OMTKY3 and a nonionizable Gln$^{18}$ OMTKY3, as a reference, is shown in Fig. 2. The behavior of log $K_f$ for the last case is extremely simple. It can be rationalized by the $K_A$ of His$^{37}$ in the catalytic triad of SGPB being 6.8 in the free enzyme and shifting to a very low value in the enzyme inhibitor complex (26, 27). It is worth noting that while the assignment of the pH dependence of Gln$^{18}$ OMTKY3-SGBP association is likely correct and interesting, it is not essential to this paper. All that is required is that the division of $K_{eq}(\text{Glu}^{18})$ and (His$^{18}$) by $K_{eq}(\text{Gln}^{18})$ eliminates the effect of all residues other than the P$_1$ residue. For Glu$^{18}$ and His$^{18}$ variants, the pH dependence is more complex, involving a combination of the effects seen in the Gln$^{18}$ variant and the $K_p$ perturbation of the P$_1$ residue by complex formation. To separate the effects, consider,

$$K_p = K_p^0 \frac{Q_p}{Q_p^*} \quad (\text{Eq. 1})$$

where $K_p$ is at arbitrary pH, $K_p^0$ is at very low pH where all the groups in the complex, inhibitor, and enzyme are fully protonated. $Q_p$, $Q_p^*$, and $Q_p^p$ are the protonation state partition functions, often called binding polynomials (28), but, in this case, probably better referred to as proton release polynomials (29–31). $Q_p^p$ is obtained from a nonionizable P$_1$ variant such as Gln$^{18}$ OMTKY3. Similarly, since the P$_1$ side chain in the complex with SGPB is insulated in a hydrophobic S$_1$ cavity (11), it seems plausible to write,

$$Q_p^p = Q_p^p(1 + \frac{K_{ion_c}}{[H^+]}) \quad (\text{Eq. 3})$$

where $K_{ion_c}$ is the ionization constant of the P$_1$ residue in the complex. Simple division now yields as follows.

$$R = \frac{K_p^{\text{ionizable}}}{K_p^{\text{nonionizable}}} = \frac{R^0 + 1 + \frac{K_{ion_c}}{[H^+]}}{1 + \frac{K_{ion_p}}{[H^+]}} \quad (\text{Eq. 4})$$

The derivation of this relation is based on a simple assumption that the ionizable P$_1$ residue is not interacting with any other ionizable group in the free inhibitor or in complex. This assumption is supported by the published x-ray structure of ovomucoid third domains (7) and by the unpublished structure of Glu$^{18}$ OMTKY3 in complex with SGPB. For plotting and fitting of the data the logarithmic form of this equation has been used,

$$\log R = \log R^0 + \log(1 + 10^{(pH - pK_i)}) - \log(1 + 10^{(pH - pK_c)}) \quad (\text{Eq. 5})$$

where $R^0$ is the equilibrium constant ratio of the fully protonated to the nonionizable residue and $pK_i$ and $pK_c$ are the pK values in the free inhibitor and in the complex with SGPB. The plots of log $R$ versus pH are shown in Fig. 3. The excellent leveling off for the His$^{18}$ and Glu$^{18}$ curves, both on the low and high pH side, clearly indicates that, around pH 3, the P$_1$ side
chain in complex and in the free inhibitor is predominantly in the Hiso and Gluo forms, both in the free inhibitor and in complex. The plots in Fig. 3 are fitted by nonlinear least squaring (32) using $pK_a$, $pK_c$, and $R^6$ as fit parameters. For Glu$^{18}$ $pK_a$ is 4.46 ± 0.05, $pK_c$ is 8.74 ± 0.06, and $R^6$ is 13.9 ± 1.3. The large 4.3-unit $pK$ shift shows that (a) both in the crystal structures and in measurements at pH 8.3, the side chain of Glu$^{18}$ is predominantly in the Gluo form, (b) binding of Gluo is 16,000 times stronger than binding of Glu$^o$, and (c) binding of Gluo is 14 times better than binding of Glu. A simple illustration of the $pK$ shift of Glu in Glu$^{18}$ variant upon complex formation with SGPB and the associated changes in $K_a$ are shown diagrammatically in Fig. 4. For His$^{15}$ and Glu$^{18}$, $pK_a$ is 6.63 ± 0.08, $pK_c$ is 4.31 ± 0.07, and $R^6$ is 0.027 ± 0.003. The downward shift of the His$^{15}$ $pK$ is consistent with avoidance of a charged form in the S1 cavity, albeit now only by a factor of 160. The binding of His$^{15}$, in its neutral form, is 4.5 times better than that of Glu$^{18}$; this was already known from the pH 8.3 measurements (18, 19). Note the striking and unforced agreement between $pK_a$ of both side chains with the expected or model values. The excellent fit of the curves to Equation 5 and the clear evidence that measurable association still takes place at the low pH limit of Fig. 3 for the His$^{15}$ variant, and at the high pH limit for the Glu$^{18}$ variant, argue for the validity of the model. The correctness of this treatment is also strongly validated by the ratio of two nonionizable side chains, i.e. $(K_a(\text{Leu}^{18}))/K_a(\text{Gl}n^{18})$ being independent of pH in the pH range 4–8.3 (Fig. 3).

The values of $K_a$ for variants with ionizable side chains that were reported (18, 19) at pH 8.3 refer to the equilibrium mixtures of the ionized and unionized forms of the P$_1$ side chain both in the free inhibitor and in the complex. In the specific case of Glu$^{18}$ variant interacting with SGPB, the free side chain is overwhelmingly Glu$^o$, while the bound side chain is largely Gluo. It is of interest to calculate the $K_a$ values for Glu$^o$ in the free inhibitor binding to form a complex with Gluo and for Glu$^o$ in the free inhibitor forming a complex with Gluo. The constants are $K_a(\text{Glu}^{18}) = 1 \times 10^{10}$ M$^{-1}$ and $K_a(\text{Glu}^{18}) = 6 \times 10^5$ M$^{-1}$ (see Fig. 4). The $K_a$ (Glu$^o$) is the fourth strongest of $K_a$ values (after Leu$^{18}$ (5.6 × 10$^{10}$ M$^{-1}$), Met$^{18}$ (2.7 × 10$^{10}$ M$^{-1}$), and (Asp$^{18}$) (2.0 × 10$^{10}$) of all 20 coded amino acid residues. At low pH (pH 4.5 or lower), the Glu$^{18}$ variant is one of the most effective inhibitors of SGPB. It is possible that the frequently seen P$_1$ Glu residues in natural protein inhibitors of serine proteinases serve not only to inhibit glutamic and aspartic acid-specific enzymes such as Glu-specific S. griseus proteinase (33) but also at low pH values to inhibit the widely distributed enzymes with hydrophobic S$_1$ pockets.

While the method is fairly labor intensive, we believe that it could be readily extended to more, possibly all, ionizable side chains (Asp and Lys seem particularly good) and to more enzymes with hydrophobic S$_1$ pockets, e.g. SGPA and chymotrypsin. Extension to enzymes, such as trypsin or Glu-specific S. griseus proteinase, with ionizable residues in their S$_1$ pockets may require more complex interpretation.

Ionizable side chains in proteins in their protonated form are either uncharged (Asp, Glu, Cys, Tyr) or cationic (His, Lys, Arg) (34). Ionization of uncharged acids yields charged products. Therefore the large upward $pK$ shift, seen upon addition of organic cosolvents to such acids, is expected (35–37). On the other hand, ionization of cationic acids involves a transfer of charge from the cationic reactant to the hydronium ion, which is one of the products. Therefore, cosolvent effects on $pK$ are anticipated to be, and are, small and erratic (35–37). Burial in a protein differs from transfer to an organic solvent, as the hydronium ion resides in an aqueous solution; only the side chain is buried. As a result, opposite effects, about half as great in absolute value as those observed in the transfer of an uncharged acid to an “equivalent” organic solvent, should be expected if the S$_1$ pockets were purely isotropic. Clearly, they are not, and dipole and charge effects may further affect the observed values.

Since this communication was first submitted, we have determined the pH dependence of the interaction between SGPB and Glu$^{18}$ OMTKY3 and found that $pK_a$ is 4.40 ± 0.10 and $pK_c$ is 9.26 ± 0.10. Therefore, the shift is 4.9 units, somewhat greater than for Glu$^{18}$. Recently (38) it was shown that Asp$^{26}$ in thioredoxin has a $pK$ of 7.5 in the oxidized form and greater than 9 in the reduced form, another huge $pK$ shift for an Asp residue. Huang and James (3) completed their high resolution structures of Asn$^{18}$ OMTKY3 at pH 6.5 and of Asp$^{18}$ Glu$^{18}$, and Glu$^{18}$ variants at pH 6.5 and 10.7. An extended joint manuscript is now readied for publication.

REFERENCES

1. Born, M. (1920) Z. Phys. 1, 45–49
2. Dao-pin, S., Anderson, D. E., Baase, W. A., Dahlquist, F. W., and Matthews, B. W. (1991) Biochemistry 30, 11521–11529
3. Langelsetmo, K., Fuchs, J. A., Woodward, C., and Sharp, K. A. (1991) Biochemistry 30, 7609–7614
4. Sittites, W. E., Gittis, A. G., Lattman, E., and Shortle, D. (1993) J. Mol. Biol. 221, 1–14
5. Yang, A.-S., and Honig, B. (1992) Curr. Opin. Struct. Biol. 2, 40–45
6. Laskowski, M., Jr., and Kato, I. (1980) Annu. Rev. Biochem. 49, 593–626
7. Bode, W., and Huber, R. (1992) Eur. J. Biochem. 204, 433–451

3 K. Huang, W. Lu, M. A. Qasim, S. Anderson, M. Laskowski, J. R., and M. N. J. James, manuscript in preparation.
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8. Schechter, I., and Berger, A. (1967) Biochem. Biophys. Res. Commun. 27, 157–162
9. Read, R. J., and James, M. N. G. (1986) Proteinase Inhibitors (Barrett, A. J., and Salveson, G., eds) pp. 301–316, Elsevier Science Publishers B.V., Amsterdam
10. Ardelt, W., and Laskowski, M., Jr. (1985) Biochemistry 24, 5313–5320
11. Huang, K., Lu, W., Anderson, S., Laskowski, M., Jr., and James, M. N. G. (1995) Protein Sci. 4, 1985–1997
12. Laskowski, M., Jr., Kato, I., Ardelt, W., Cook, J., Denton, A., Empie, M. W., Kehr, W. J., Park, S. J., Parks, K., Schatzley, B. L., Schoenberger, O. L., Tashiro, M., Vichot, G., Whatley, H. E., Wieczorek, A., and Wieczorek, M. (1987) Biochemistry 26, 202–221
13. Apostol, I., Giletto, A., Komiyama, T., Zhang, W., and Laskowski, M., Jr. (1993) J. Protein Chem. 12, 419–433
14. Fujinaga, M., Read, R. J., Sielecki, A., Ardelt, W., Laskowski, M., Jr., and James, M. N. G. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 4868–4872
15. Read, R. J., Fujinaga, M., Sielecki, A. R., and James, M. N. G. (1983) Biochemistry 22, 4420–4433
16. Fujinaga, M., Sielecki, A. R., Read, R. J., Ardelt, W., Laskowski, M., Jr., and James, M. N. G. (1987) J. Mol. Biol. 195, 397–418
17. Bode, W., Wulff, A.-Z., Huber, R., Meyer, E., Travis, J., and Neumann, S. (1986) EMBO J. 5, 2453–2458
18. Bigler, T., Lu, W., Park, S. J., Tashiro, M., Wieczorek, M., Wynn, R., and Laskowski, M., Jr. (1993) Protein Sci. 2, 786–799
19. Lu, W., Zhang, W., Rothberg, I., Chiang, Y., Anderson, S., and Laskowski, M., Jr. (1993) Protein Sci. 2, suppl. 122
20. Jurausk, L., Johnson, P., Ohlson, R. W., and Smillie, L. B. (1971) Can. J. Biochem. 49, 1195–1201
21. Lu, W. (1994) Energetics of the Interactions of Ovomucoid Third Domain Variants with Different Serine Proteinases: Ph.D. thesis, Purdue University
22. Green, N. M., and Work, E. (1953) Biochem. J. 54, 347–352
23. Empie, M. N., and Laskowski, M., Jr. (1982) Biochemistry 21, 2274–2284
24. Park, S. J. (1985) Effect of Amino Acid Replacement in Ovomucoid Third Domain upon Their Association with Serine Proteinases. Ph.D. thesis, Purdue University
25. Wynn, R. (1990) Design of a Specific Human Leukocyte Elastase Inhibitor Based on Ovomucoid Third Domains. Ph.D. thesis, Purdue University
26. Finkenstadt, W. R., Hamid, M. A., Mattis, J. A., Schroe, J., Sekelak, R. W., Wang, D., and Laskowski, M., Jr. (1974) in Bayer Symposium V on Proteinase Inhibitors (Fritz, H., Tschesche, H., Greene, L. J., and Truscheit, E., eds) pp. 399–411, Springer-Verlag, New York
27. Fioretti, E., Angeletti, M., Moleti, A., Bolognesi, M., Menegatti, E., Rizzi, M., and Ascoli, F. (1993) J. Enzyme Inhibition 7, 57–64
28. Wyman, J., and Gill, S. J. (1990) Binding and Linkage, pp. 33–122, University Science Press, Mill Valley, CA
29. Lebowitz, J., and Laskowski, M., Jr. (1962) Biochemistry 1, 1044–1053
30. Laskowski, M., Jr., and Finkenstadt, W. R. (1972) Methods Enzymol. 26, 193–227
31. Mattis, J. A., and Laskowski, M., Jr. (1973) Biochemistry 12, 2239–2245
32. Leatherbarrow, R. J. (1987) Enzfitter (version 1.05), Biosoft, Cambridge, U.K.
33. Komiyama, T., Bigler, T. L., Yashida, N., Noda, K., and Laskowski, M., Jr. (1991) J. Biol. Chem. 266, 10727–10730
34. Edsall, J. T., and Wyman, J. (1958) Biophysical Chemistry, pp. 47–135 and 477–549, Academic Press, New York
35. Michaelis, L., and Mizutani, M. (1925) Z. Phys. Chem. 116, 135–160
36. Halford, J. O. (1933) J. Am. Chem. Soc. 55, 2272–2278
37. Homandberg, G. A., Mattis, J. A., and Laskowski, M., Jr. (1978) Biochemistry 17, 5220–5227
38. Wilson, N. A., Barbar, E., Fuchs, J. A., and Woodward, C. (1995) Biochemistry 34, 8931–8939
39. Laskowski, M., Jr., and Finkenstadt, W. R. (1972) Methods Enzymol. 26, 193–227
40. Mattis, J. A., and Laskowski, M., Jr. (1973) Biochemistry 12, 2239–2245
41. Leatherbarrow, R. J. (1987) Enzfitter (version 1.05), Biosoft, Cambridge, U.K.
42. Komiyama, T., Bigler, T. L., Yashida, N., Noda, K., and Laskowski, M., Jr. (1991) J. Biol. Chem. 266, 10727–10730
43. Edsall, J. T., and Wyman, J. (1958) Biophysical Chemistry, pp. 47–135 and 477–549, Academic Press, New York
44. Michaelis, L., and Mizutani, M. (1925) Z. Phys. Chem. 116, 135–160
45. Halford, J. O. (1933) J. Am. Chem. Soc. 55, 2272–2278
46. Homandberg, G. A., Mattis, J. A., and Laskowski, M., Jr. (1978) Biochemistry 17, 5220–5227
47. Wilson, N. A., Barbar, E., Fuchs, J. A., and Woodward, C. (1995) Biochemistry 34, 8931–8939
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