IS THE SERINE PROTEASE "CHARGE RELAY SYSTEM" A CHARGE RELAY SYSTEM?

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Summary: The catalytic mechanism of the serine proteases as currently conceived specifies that the charge relay system Asp is ionized at pH's where the enzymes are active. This choice is questioned on the basis that external anions are found associated with the charge relay system of both chymotrypsin and subtilisin at pH's below the charge relay system pK, and no evidence has been found for the presence of an associated cation at pH's above the charge relay system pK. An alternative catalytic mechanism is proposed wherein Nδ1 of the His imidazole is hydrogen bonded to a neutral Asp, and His-Nε2 functions to relay a proton from Ser-Oγ to the substrate in a manner analogous to proton transfer in ice.

The serine protease "charge relay system" is comprised of three amino acid side chains: serine hydroxyl, histidine imidazole and aspartic acid carboxylic acid (1). The relative positions of these three functional groups are essentially the same in all of the serine proteases for which high resolution X-ray structures have been determined (2). The location of the protons associated with the charge relay system is subject to less certainty and must be determined by inference from chemical, spectroscopic and structural studies.

The protonic equilibrium shown below (I) was initially proposed by Blow et al. (1). On the basis of ^{13}C-NMR results with α-lytic protease, Hunkapiller et al. (3) postulated that the equilibrium favors the uncharged species at low pH. After reviewing data from our own laboratory and from other's, we feel that the equilibria shown in (II) must also be seriously considered. The presence of a hydrogen bond between Ser-Oγ and His-Nε2 in the native enzyme is still uncertain.

The two schemes may be expected to differ in a manner which is subject to experimental verification: at pH's between approximately 4 and 6 the charge relay system of (I) is electrically neutral whereas (II) requires an

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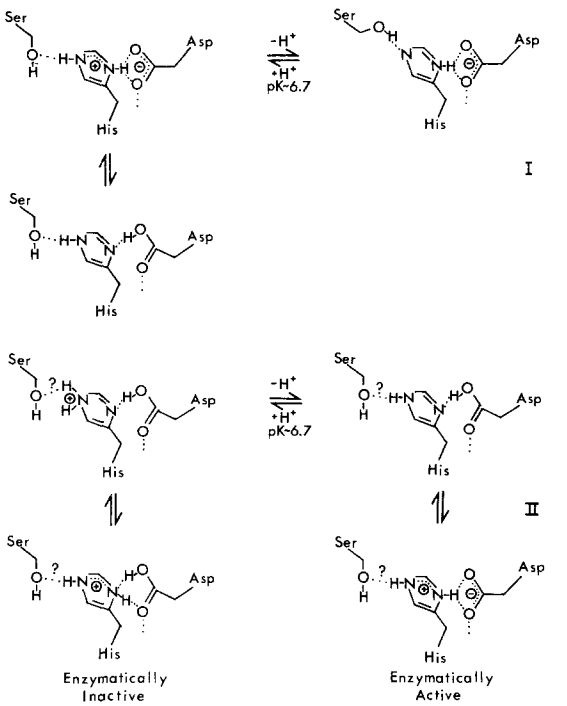


Scheme 1

external anion; at pH's above the pK of the charge relay system, (I) requires an external cation whereas (II) is electrically neutral. The charge relay system Asp residue in the native forms of both chymotrypsin and subtilisin is buried within a region of the protein where there are no protein cationic groups, other than the imidazolium ring, nor is there any extraneous electron density which could reasonably be attributed to the presence of a cation from the solvent (4,5).

Although conclusive evidence is not yet available, data which is available suggests that an external anion is associated with the charge relay system at pH's below the charge relay system pK, and that there is no associated cation at higher pH's. In α-chymotrypsin at pH 3.6 a $SO_4^{=}$ (or HSO_4^{-}) is hydrogen bonded to Ser-195-Oγ, Gly-193-N and Tyr'-146-Oη (prime denotes residue from adjacent molecule) (6). The carboxylic acid group of Tyr'-146 hydrogen bonds to the peptide carbonyl of His-57. The sulfate is centered

approximately 5Å from His-57-Nε2 and thus it probably is the counterion for the imidazolium ring at very low pH. At pH 5.4 the position of the sulfate remains unchanged but the carboxyl group of Tyr'-146, which is exposed to solvent and should be largely ionized, has moved to within hydrogen bonding distance of His-57-Nε2. A similar situation is clearly observed in two subtilisin inhibitor complexes at pH 5.9. Both N-benzoyl-L-arginine (7) and carbobenzoxyglycylglycyl-L-tyrosine (8) are found oriented in the active site with one of their carboxylate oxygens approximately 3.5Å from His-64-Nε2. It would appear unnecessary for an external carboxylate to be associated with the imidazolium ion if the charge relay system Asp were also ionized.

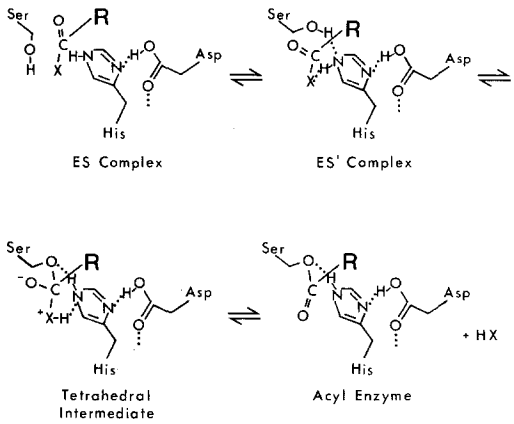
If an external cation is associated with the charge relay system at pH's >6.7 as predicted by (I), the cation must necessarily be a factor in catalysis. An intriguing possibility for the active participation of the cation in catalysis involves initial displacement of the cation by the incoming substrate molecule. The cation would then associate with the bound substrate molecule (conceptually with the most electronegative atom - the carbonyl oxygen) polarizing the carbonyl group and inducing proton transfer from Ser-Oγ via His-imidazole to Asp-Oδ1,2. With this possibility in mind, we attempted to obtain evidence for the presence of a charge relay system cation by adding Ag_2SO_4, at the maximum amount which could be accommodated without cracking the crystals, to the milieu of subtilisin BPN' crystals at pH 7.5 (9). The electron density difference maps were featureless except for several spherical regions of weak (highest contour levels 4σ) positive electron density, one of which was in the vicinity of the active site. All of the positive regions were in close proximity to surface methionine residues. The positive region at the active site was adjacent to Met-222-Sδ, more than 4Å from His-64-Nε2. Positive density was not observed in the vicinity of Asp-32, the position corresponding to the Ag^+ in Ag-DIP-trypsin (10). Ag^+ complexes readily with amines and Ag^+ would be anticipated to form a coordination complex with His-64Nε2 if Asp-32 were ionized. We cautiously submit

this observation as evidence that the charge relay system of the native enzyme does not require an external cation with the attendant implication that the charge relay system Asp is not ionized in the pH range where the enzyme is active. In further support of this possibility, large changes in ionic strength and the nature of the monovalent cation have only a small effect on both K_M and k_{cat} (11).

The possibility exists that a negative charge on the charge relay system Asp could be dissapated through the internal hydrogen bond network (4,5) of these proteins thereby obviating the necessity for a discrete cation. However, the Asp side chain is linked directly to the surface through the His-imidazole. By analogy to the relative positions of Fe(III) and its counter-ion in bis(imidazole)-tetraphenylporphinato iron (III) chloride (12) and in metmyoglobin (13), one would expect a cation associated with the charge relay system His-Nε2 under conditions where a net negative charge resides in the Ser, His, Asp triad.

A seemingly unique low-field proton resonance associated with the charge relay system His and Asp side chains of both δ-chymotrypsin and chymotrypsinogen was reported by Robillard and Shulman (14). At pH 4.5 this resonance occurred at 18 ppm downfield from DSS, shifting upfield as a function of pH to 15 ppm with an apparent pK of approximately 7. In the absence of analogous spectra for simple model compounds, it is difficult to predict which of the two protonic equilibria is in better accord with the NMR result. It does not appear that either can be presently excluded on this basis. In both (I) and (II) the Asp, His protons at low pH are more highly deshielded than the proton(s) at pH's above the pK.

Although additional evidence can be cited in support of (II) versus (I) (15), the case is not compelling. An important consideration in deciding between the two alternatives is which protonic equilibrium leads to a catalytic mechanism more consistent with the large body of chemical, spectroscopic and structural data on these enzymes. Accordingly, we suggest the mechanism



Scheme 2

diagrammed in brief form below. Hydrolysis of the acyl enzyme involves a H_2O molecule occupying the site vacated by HX and reversal of steps leading to the acyl enzyme. In respect to the proton transfer steps, the mechanism is similar to that proposed initially by Parker and Wang (16). The essential difference is that here the His-Nε2 functions in proton transfer exactly like the oxygens in ice (17). To function in this manner His-Nε2 need not be a particularly strong base, but only need be positioned precisely in the ES complex relative to Ser-Oγ and the atom of the substrate receiving the proton. Thus the primary function of the Asp side chain in the serine protease triad may be to orient precisely the imidazole ring and not to increase the basisity of the imidazole, in which case the "charge relay system" does not relay a charge but the imidazole alone relays a proton.

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