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# ON THE INACTIVITY OF THIOL-SUBTILISIN. THE ROLE OF THE INTRAMOLECULAR ELECTRIC FIELD

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Based on computed proton affinities for several model systems, the energetics of proton transfer and the acidity of the catalytic triads Cys–His–Asn (papain), Cys–His–Asp (thiol-subtilisin) and Ser–His–Asp (subtilisin) are discussed. It is shown that in papain the ion-pair $Cys^-$–$HisH^+$ exists owing to the intramolecular electric field, and that a similar situation is found in thiol-subtilisin, but not in subtilisin. Assuming similar reaction mechanisms for papain and thiol-subtilisin – i.e. proton transfer from $HisH^+$ to the NH group of the scissile peptide bond – the inactivity of thil-subtilisin towards proteins is explained by the much greater basicity of His in the complex His–$Asp^-$ than in His–Asn. In order for this explanation to be consistent, it is tentatively concluded that the catalytic mechanism of the serine proteases is different from that of the cystein proteases, and involves direct transfer of the serine proton to the leaving group in the acylation step.

## 1. Introduction

In 1966 thiol-subtilisin was synthesized, almost simultaneously, by Neet and Koshland [1] and by Polgar and Bender [2]. Thiol-subtilisin (SHSTL) differs from the native enzyme subtilisin (STL) in that the oxygen atom of the active site serine is replaced by sulfur. More or less surprisingly, this substitution destroys protease activity completely [1,3] and greatly reduces activity towards other substrates [1–4].

From the three-dimensional structure of STL [5–7] we know that the active site comprises the triad Ser221–His64–Asp32, which in SHSTL is turned into the triad Cys221–His64–Asp32. One now can think of several reasons for the inactivity of SHSTL.

i) The combination Cys–His is not a good active site.

This assumption is contradicted by the existence and activity of papain [8] and actinidin [9] and several other cystein proteases [10].

ii) The substitution of OH by SH severely distorts the enzyme's structure.

The early investigators [1–4] made sure that the chemical treatment did not change the physico-chemical properties of STL, and found similar properties for SHSTL. Recent kinetic studies [11,12] on STL and SHSTL with specific substrates lead to the conclusion that the active sites are very similar. Also, X-ray analysis [13] showed – apart from changes due to the O,S substitution – identical structures for STL and SHSTL.

iii) The larger atomic radius of S prevents proper stabilization of the tetrahedral intermediate, because the substrate's carbonyl oxygen no longer fits into the oxyanion hole [14].

This possibility deserves serious attention. However, the C–S–C angle is smaller than the C–O–C angle. Bending modes are essentially soft, and thus a slight further decrease in bond angle might well compensate the increase in bond length of 0.4 Å, in which case the carbonyl oxygen is back into its original position. Moreover, we do not know how critical this fit is in relation to proper stabilization of the intermediate.

iv) The triad Cys–His–$Asp^-$ does not form a good active site in the subtilisin structure.

This paper concerns this last possibility. We note that in the discussion of enzyme mechanism, it is rather common to concentrate on the active site's con-

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stituents *in isolation*. The active site's environment then is only mentioned in relation to possible formation of hydrogen bonds etc., the rest of the enzymes three-dimensional structure only in connection with the non-covalent enzyme/substrate complex. Recently, we reported *ab initio* calculations [15,16] on systems modelling the active site of papain (PAP). It was shown that the electric field generated by the active site helix [17] has considerable influence on the equilibrium position of the essential proton [15] as well as on the proton affinities of the active site residues [16].

Here, we compare the triads Cys--His--Asn and Ser--His--Asp of PAP and STL, respectively, with the triad Cys--His--Asp of the synthetic SHSTL and of hypothetic "aspartate-papain". Rather than using expensive calculations we discuss, in terms of calculated proton affinities and environmental effects obtained earlier [15,16] the energetics of proton transfer in these triads, as well as the possible consequences for the reaction mechanisms of cystein and serine proteases.

## 2. Experimental observations

Recent investigations of Tsai and Bender [11] and of Pillip et al. [12], comparing the activity of STL and SHSTL towards specific substrates, confirmed the early findings [1–4]. Inhibition with carbobenzyl-oxy-L-alanylglycyl-L-phenylalanine [11] showed that alkylation of SHSTL takes place at sulfur (like in PAP) rather than at imidazole (like in STL). Acylation with p-nitrophenylbutyrate [12] showed for SHSTL a bell-shaped pH profile with inflexion points at 6.1 and 8.5, where STL has only one at pH 7.1. For deacylation, both STL and SHSTL have a single inflexion point at pH 7.1, but SHSTL is less active than STL.

Acylation of PAP shows bell-shaped pH dependence [10] with inflexion points at pH 4.2 and 8.2. Deacylation shows sigmoidal pH dependence with pK about 4. Lewis et al. [18] reinvestigated this behaviour for pH below 6 and concluded that in this region activity depends on two groups, with pK 3.8 and 4.0, respectively. Zannis and Kirsch [19] investigated substituent effects on deacylation of acyl-papains and concluded that a group of pK 4 (Asp158?) is involved, while the tetrahedral intermediate carries a charge of −1.

Table 1
Proton affinities (kcal/mol) with references

| | calculated | experimental |
|---|---|---|
| $CH_3S^-$ | 363 [16] | 337 [20] |
| Imidazole (Im) | 242 [16] | 222 [20] |
| $CH_3O^-$ | 411 [21] | 408 [21] |
| $HCOO^-$ | 358 [21] | 242 [22] |
| $NH_3$ | 222 [21] | 207 [23] |
| $[CH_3S ... Im]^-$ | 337 [16] | – |
| $[CH_3SH ... Im]$ | 173 [16] | – |

## 3. Intramolecular field and the energy of proton transfer

In table 1 proton affinities are listed for some species relevant to the present problem. They are obtained from fairly accurate *ab initio* quantum mechanical calculations. Since many experimental values for proton affinities are obtained from rather intricate experiments or from comparison with related systems [20] we will use the theoretical values. These are all connected with simple removal or addition of a proton *in vacuo* and, hence, will all contain about the same error. This should make computed differences more reliable.

Fig. 1 displays schematically the triads found in the active sites of many proteolytic enzymes, with appropriate distances as found in experimental structures.

Table 2 gives an analysis of the energy effects associated with the various proton transfer processes. For computing the coulomb interactions between ionic moieties, the (unit) negative charges were situated on X (O in Ser, S in Cys) and halfway between O and Y (O in Asp, $NH_2$ in Asn), the (unit) positive charge of the imidazolium ion on N. The entries labeled "protein field" are an estimate, taken from the papain model calculations [15,16] and are largely due to the effect of

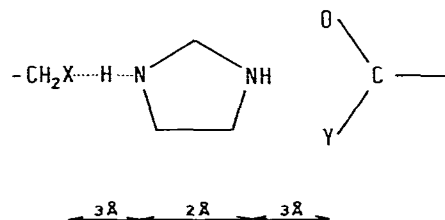


Fig. 1. Schematic representation of active site triads. X = S in Cys, X = O in Ser. Y = $NH_2$ in Asn, Y = O in Asp.

Table 2
Energies of proton transfer (kcal/mol)

| | $Ser^0His^0Asp^-$ ↓ $Ser^-His^+Asp^-$ | | $Ser^0His^0Asp^-$ ↓ $Ser^-His^0Asp^0$ | | $Cys^0His^0Asn$ ↓ $Cys^-His^+Asn$ | | $Cys^0His^0Asp^-$ ↓ $Cys^-His^+Asp^-$ | |
|---|---|---|---|---|---|---|---|---|
| proton affinities | Ser | +411 | Ser | +411 | Cys | +363 | Cys | +363 |
| | His | −242 | Asp | −358 | His | −242 | His | −242 |
| coulomb interactions [a] | $X^-$, $His^+$ | −110 | | | | −110 | | −110 |
| | $His^+$, $Y^-$ | −66 | | | | | | 66 |
| | $X^-$, $Y^-$ | +42 | | | | | | +42 |
| transfer energy (vacuum) | | +35 | | +53 | | +11 | | −11 |
| protein field [b] | | −20 | | −20 | | −20 | | −20 |
| transfer energy (in protein) | | +15 | | +33 | | −9 | | 33 |

[a] For distances between charges, see fig. 1. 1e/Å = 332 kcal/mol.
[b] Based on the electric field between X and N in papain and subtilisin [15–17].

the active site helix in papain. Since in PAP and STL about the same protein field was found [17] the same value was used throughout.

According to table 2, in the naturally occurring triads *in vacuo* proton transfer from X to imidazole is not to be expected, it being endothermic by 35 kcal/mol in Ser–His–Asp$^-$ and by 11 kcal/mol in Cys–His–Asn. (Our *ab initio* calculations on the model systems for papain [15,16] gave values of about 15 kcal/mol. From this we may estimate the accuracy of the present arguments).

Taking into account the protein field, we see that in the triad Cys–His–Asn the ion pair is stabilized to an extent that we may expect this configuration to be the more stable one in PAP and in actinidin. The value of 20 kcal/mol for the effect of the protein is probably a lower limit, because in our calculations we used the smallest possible value for the helix dipole field [17], and we did not incorporate possible polarization effects [24,25], which are always stabilizing.

The existing physico-chemical evidence for the presence of an ion-pair in PAP [26–30] was critically reviewed by Lowe [10]. He concludes: "The question remains as to where *exactly* the proton is ... ". Also Zannis and Kirsch [19] conclude that the evidence is insufficient to decide the existence of an ion pair. They advocate the importance of Asp158 in the mechanism of papain, but want "more precize physical measurements on single residues without the necessity of chemical perturbance".

Good quality quantum mechanical calculations provide just the sort of information these authors want, and strongly support the existence of the ion-pair in papain. Whether or not Asp158 is ionized [18,19] is not very important for the stability of the ion pair. Considering only coulombic interactions, and realizing that the carboxylate group is 7Å away from both S and N, the negative charge will stabilize the proton to the same extent in either position.

In the Ser–His–Asp triad, however, stabilization by the field is insufficient for creating an ion-pair. Also, in the serine proteases, double proton transfer – often suggested for the charge relay system [21,31,32] – is not very likely to occur. Basically because the negative ion lacks coulomb stabilization, the double transfer is almost as endothermic as single proton transfer without the field. Moreover, a detailed analysis of the protein field in the region between His and Asp [20,33] reveals that it certainly does not enhance transfer of the second proton.

Turning to the triad Cys–His–Asp$^-$ we conclude that this will almost certainly form an ion-pair, because the negative charge on Asp – not present on Asn in PAP – stabilizes the imidazolium ion by an amount compar-

able to that by the protein field, making the zwitterion already stable in *vacuo*. (For comparison: our estimate of an additional stabilization of 24 kcal/mol is close to the 18 kcal/mol found by Hayes and Kollman [20]) Again, the results of our calculations seem to confirm earlier suggestions made on experimental grounds for SHSTL [34].

## 4. Protolytic equilibria

The assignment of the relevant pK values to active site residues in PAP has been subject of many discussions. Accepting the ion-pair configuration on the basis of our computed results, we now follow Drenth et al. [30] and assign a pK ≈ 4 to Cys, pK ≈ 8 to His in the free enzyme, and pK ≈ 4 to His in the enzyme with a blocked thiol group. For reasons of easy reference we repeat here the arguments formulated recently [16]. The calculated proton affinity of $[CH_3 \ldots Im]^-$ in the protein field (table 1) is close to that of free $CH_3S^-$. Owing to the stability of the ion pair (table 2) the first proton will be attached to His, which then should exhibit a basicity close to that of free Cys (pK ≈ 9). The computed proton affinity of $[CH_3SH \ldots Im]$ is much smaller than that of free His, and hence this group should be less basic than His (pK ≈ 6). Since the only place for the proton to go is Im, a value <6 must be assigned to His in this case.

Proton affinities are not easily related to pK values. If we look, for example, at the values for $CH_3S^-$ and $HCOO^-$ in table 1, blind application of the rule: "like PA's, like pK's" would lead to the erroneous result that methanethiol and formic acid should have the same acidity. However, the values in table 1 refer to systems excluded from (bulk) water. Presumably, the situation in the active site of an enzyme is not very different from a (rather dense) gas phase, i.e. rather hydrophobic [10]. We note that accurately computed proton affinities and interactions under such conditions compare very well with values obtained from gas phase experiments. (For a recent review on hydrogen bonded systems, see e.g. [35]).

With these assumptions, the peculiar pK values found for PAP are easily explained. The helix field pushes the proton from Cys to His, thus lowering the pK of the former and increasing that of the latter residue. Another example of a cysteine with a very low pK (≈6) is found in rhodanese [36] where this residue also is situated at the N-terminal and of a helix [37,38]. By taking away the negative charge on the sulfur atom – either by putting a proton next to it like in the theoretical model, or by attaching some other group like in experiment – the imidazolium ion is destabilized and will now have much less tendency to keep its proton. Hence we feel that the calculations also support the assignment of pKs in papain.

Applying the same reasoning to the Cys–His–$Asp^-$ triad in SHSTL and to Ser–His–Asp in the serine proteases, we expect similar behaviour, i.e. a relatively low pK for Cys (Ser) and a high pK for His in the free enzymes. For SHSTL this is in accord with experiment: acylation is pH dependent in a way similar to PAP [12, 34]. The shift of the typical bell shaped curve towards larger pK values for SHSTL [34] relative to PAP is understandable by taking into account the influence of $Asp^-$ in the active site of SHSTL where PAP has only neutral Asn.

In the serine case, there will always be a proton on the oxygen (the "low" pK of Ser being still well above 10), i.e. we must compare it with $[CH_3SH \ldots Im]$ in our model system or with PAP with a blocked thiol group, where His has a much smaller pK than in the free enzyme. Indeed, SHSTL with the sulfur atom engaged in a covalent bond, free STL and acylated STL all show the same smaller pK for His than free SHSTL [11,12,34]. The fact that this pK is not lowered to a value of 4 – like in PAP – again can be attributed to the close proximity of $Asp^-$, which still stabilizes the imidazolium ion when the negative charge (on Cys in SHSTL) is gone, or never exists (on Ser in STL).

## 5. Mechanistic consequences

It is now well established that the active site of PAP is zwitterionic. The proposed reaction mechanism [30] involves proton tranfer from $HisH^+$ to the NH group of the scissile peptide bond, a step which is probably rate determining [39]. Whether or not a tetrahedral intermediate is formed – which is widely accepted, but for the serine proteases put to question by Komiyami and Bender [30] – the engagement of the substrate's carbonyl carbon in a covalent bond with the enzyme's nucleophile will make the NH group act as an amine, rather than as an amide. Consequently, it

will be more basic than $NH_3$, and since the proton affinity of His tends to become smaller when the sulfur atom looses its negative charge, this proton transfer will be easier. The numbers in table 1 seem to agree with such a mechanism.

SHSTL, according to the arguments presented in the foregoing section, most likely also has the ion-pair configuration. Then, in order to be effective, it should have a mechanism like that of PAP, i.e. the rate limiting step with proteins should be acyl-enzyme formation by disrupting the peptide CN bond through protonation of the NH group. However, in view of the energetics given in section 3, this is hardly possible since the proton in $HisH^+$ $-Asp^-$ is bound too tightly. Hence, no acyl-enzyme will be formed, as was already noted by Neet et al. [3].

In other words, according to the papain mechanism, protonation of the scissile peptide bond NH-group requires a His with $pK \approx 4$, while in SHSTL (and STL) it is as large as 7. (The experiments reported in refs. [11] and [12] all refer to acyl-enzymes formed directly, and this argument does not easily enter the discussion there). Calculation of the electric field in the region between imidazole and the leaving group in both STL and PAP [41] shows that no substantial contribution of the field exists there. Hence, it is indeed the difference in the catalytic triads that is responsible for the different behaviour. Sofar, this accounts for the inactivity of SHSTL towards proteins.

As pointed out by a referee, the larger basicity of His in SHSTL as compared with PAP, should make the latter enzyme slower in the (assumed general base catalyzed) deacylation step. Comparing two identical situations – deacylation of indoleacryloyl (IA) PAP [42] and IASHSTL [11], both in 30% dioxan – we see, indeed, that papain is about an order of magnitude slower than thiol-subtilisin.

If we accept the foregoing rationalization of the inactivity of SHSTL, i.e. the proton in $HisH^+$–$Asp^-$ being bound too tightly, the same must hold for any such pair as they are found in the serine proteases. As a consequence, these proteases should have a reaction mechanism different from that of the cystein proteases, i.e. without complete proton transfer to His in the free enzymes. The simple energetics of section 3 support this view: ion-pairs $Ser^-$--$His^+$ are unstable and complete proton transfer is very unlikely, even in the presence of a substrate. Also, formation of a hydrogen bond between Ser and His is not to be expected, since a recent refinement of the structure of STL [43] showed an Oγ(221)--Nε2(64) distance of 3.7 Å. There is every reason to believe that such large distances are also found in the other serine proteases [44]. Moreover, in STL Oγ is not in its ideal position for hydrogen bonding [43].

Hence, we suppose a mechanism for the serine proteases in which the proton moves directly from Oγ to the N atom of the leaving group, more or less in a way suggested by Komiyami and Bender [40]. The His--$Asp^-$ pair is probably needed for creating a channel for the proton to move away from Oγ, and -- following Matthews et al. [44] -- forming a hydrogen bond with the newly formed amine group. His alone or His Asn is insufficient, while His - Asp in SHSTL is overdoing this, making the latter inactive towards proteins.

## 6. Discussion

The ideas presented in the foregoing sections are based on proton affinities obtained from fairly accurate quantum mechanical calculations on simple as well as more complicated model systems. In the latter case, special attention was paid to the influence of the active site's environment [15,16]. The intramolecular fields produce shifts in physical properties which may confuse the interpretation of experimental results. Advanced kinetic techniques combined with accurate three-dimensional structure determinations have greatly improved our knowledge of enzymes. However, X-ray structures do not reveal proton positions, and kinetic evidence -- of necessity – results from fitting data to model equations. On several occasions [10,19] authors formulated the need for more independent information. Good quality quantum mechanical calculations – as possible with today's electronic computers – to our esteem can provide such information. As input, no more (nor less!) than a reasonably accurate X-ray structure is needed, and no further assumptions are required. Although the actual computations are of a complicated nature, the results may be interpreted in rather simple terms -- e.g. electrostatic effects on conformational energies, etc. -- which allow easy transfer to related systems.

Within this framework, the arguments given before

provide a straightforward explanation for the inactivity of SHSTL towards proteins. Admittedly, this explanation heavily rests on the assumption that papain, under normal conditions, has the ion-pair configuration and associated mechanism. Recently, it has been argued that Asp158 could be essential in this catalytic mechanism. Lewis et al. [18] – although accepting the ion pair – found dependence of $k_{cat}/K_M$ on two pKs below 6. Zannis and Kirsch [19] have reservations about the ion pair and suggest direct involvement of Asp158, in particular in the deacylation step. They admit that the large distance from the active site (about 7 Å) does not permit direct interaction without drastic conformational changes. For pure electrostatic reasons the carboxylate group of Asp158 (carrying a full minus charge under normal conditions) will certainly influence the ionization equilibria of nearby groups, even at a distance of 7 Å, and this may well be one of its functions. However, it is interesting to compare the primary structures of papain, ficin and stem bromelain (see e.g. ref. [10] and references cited there). In the first two enzymes we find, in the amino end of the chain, Ser24–Cys25–Trp26, where bromelain has Ala24 instead of Ser. In the carboxyl end of the chain a similar change is seen, papain and ficin having Asp158–His159–Ala160 where bromelain has Asn158 instead of Asp, as was only recently found by Goto et al. [45]. Combined with the remark [10] that bromelain has a specificity different from that of papain and ficin – which are supposed to be similar – this suggests that residue 158 in these enzymes has more to do with substrate binding, as already suggested by Drenth et al. [46], than with the actual hydrolysis.

Assuming also for SHSTL the ion pair arrangement and mechanism, our conclusion in this particular case is that an essentially "good" active site pair (Cys–His) is placed in the wrong environment ($Asp^-$, plus the rest of the subtilisin structure). More generally, the inactivity of SHSTL emphasizes the notion that enzymes have structures, carefully designed to support a particular mechanism. This is also apparent in the constancy of the active site triads (Ser–His–Asp in the serine proteases, Cys–His–Asn in the cystein proteases) and examplified in the identical backbones and active sites of papain and actinidin, which have different primary structures.

More tentatively, but consistent with our rationalization of the inactivity of SHSTL, we arrive at the conclusion that the mechanism of serine proteases is different from that of the cystein proteases. Both our calculations on the stability of the $HisH^+$–$Asp^-$ pair, and the recent structural information showing rather large O–N distances in serine proteases, suggest a mechanism in which the serine proton is transferred directly to the leaving group in the acylation step of peptide hydrolysis. However, more accurate computations are needed in the investigation of this possibility, and we hope to undertake such work in the near future.

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