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On the pH dependence of thermodynamic stability of α-amylase inhibitor tendamistat

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Abstract

In this study the pH dependence of the thermodynamic stability of tendamistat is analyzed. This small globular protein of 74 residues shows a very marked dependence of thermal stability on pH: the denaturation temperature increases from 68.9°C at pH 2.0 to 93.2°C at pH 5.0, and then decreases to 77.8°C at pH 8.0. Analysis of the data indicates that the binding of two protons is coupled to the thermal unfolding at pH values below 4.0, whereas one proton is released by the protein at pH values above 5.0. By linking the proton binding to the conformational unfolding equilibrium, a thermodynamic model, which is able to describe the dependence upon the solution pH of the denaturation Gibbs energy change for tendamistat, is developed.

Keywords: Thermodynamic stability; Proton binding; Thermal unfolding; Linkage relationships

1. Introduction

The thermodynamic stability of globular proteins is significantly affected by the solution pH [1–4], indicating that ionizable groups play an important role in protein stability. The traditional explanation follows the theoretical model developed by Linderstrom-Lang [5]: the native protein is considered as a sphere with positive and negative charges distributed on the surface. At the isoelectric point of the protein there is perfect balance among negative and positive charges, whereas on decreasing or increasing the solution pH from the isoelectric point there is an unbalance. The latter situation, clearly, gives rise to electrostatic interactions that tend to destabilize the native structure. Therefore, the maximum stability of a protein should occur at its isoelectric point where the net charge is zero; such prediction has been verified in several cases [4–5].

However, there is increasing evidence that a significant fraction of the electrostatic contribution to the Gibbs energy difference between native and denatured states is due to a small number of amino acid residues whose pK_a values are shifted anomalously in the folded conformation [6,7]. At acid pHs the destabilization is caused by the protonation in the unfolded state of acid groups possessing very low pK_a values in the folded conformation, whereas at basic pHs the destabilization is caused by the release of protons in the unfolded state from basic groups possessing very high pK_a values in the folded conformation. A deeper

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understanding of the pH dependence of the stability of globular proteins is important, since electrostatic interactions, and salt-bridges in particular, seem to play a fundamental role in thermophilic proteins [8]. Moreover, Dahlquist and co-workers, by means of NMR measurements and site-directed mutagenesis, were able to show the existence, in the native structure of T4 lysozyme, of a salt-bridge between the side-chains of His31 and Asp70, with pK_a values of 9.1 and 0.5, respectively [9]. This salt-bridge stabilizes the folded conformation by about 15 kJ mol^{-1} at room temperature, which is a very large quantity.

The α-amylase inhibitor tendamistat is a small globular protein consisting of 74 residues (8 kDa), isolated from *Streptomyces tendae 4158*. It has two disulfide bridges between Cys11–Cys27 and Cys45–Cys73, which create two non-overlapping loops of significantly different size. The native structure of tendamistat has been solved both in single crystals by X-ray diffraction analysis [10], and in solution by NMR [11]. There is a very good agreement between the atomic coordinates determined by the two methods, with only minor differences for side-chains on the surface of the molecule [12]. The protein has a compact structure with two β-sheets, each consisting of three anti-parallel strands, packed against each other, forming a barrel. The strands are arranged in the following manner: I (residues 11–17), II (residues 19–26) and V (residues 51–57) in one sheet; III (residues 30–37), IV (residues 40–49) and VI (residues 64–73) in the other sheet. This topology corresponds to the immunoglobulin fold [10]. β-turns occur at residues 17–20, 37–40 and 49–52. The *N*-terminal segment from Ser5 to Pro9 assumes a polyproline II conformation. The three residues Trp18-Arg19-Tyr20 are solvent-exposed and the two aromatic side-chains are stacked; these features are related to their involvement in the interaction with α-amylase [12].

A detailed study on the thermal stability of tendamistat, by means of differential scanning calorimetry (DSC), was carried out by Hinz and co-workers [13]. The denaturation process proved to be reversible in the pH range 2.0–8.0, and well-represented by the two-state N ⇔ D transition model. Interestingly, the protein stability was markedly dependent upon pH: in the pH range 2.0–5.0, there is an increase of about 25°C in the denaturation temperature, while in the pH range 5.0–8.0 it decreases by 15°C [13]. Such pH dependence has not been carefully analyzed and rationalized until now. The aim of the present work is to perform such a task. Analysis of experimental DSC data points out that an uptake of almost two protons is coupled to the thermal unfolding of tendamistat at pH values below 4.0, whereas the release of one proton occurs at pH values above 5.0. A thermodynamic model, which links the proton binding to the conformational unfolding transition, is able to reproduce the pH dependence of the denaturation Gibbs energy change.

2. Analysis of experimental DSC data

Hinz and co-workers performed DSC measurements on tendamistat in the pH range 2.0–8.0 and 10 mM phosphate buffer: in these experimental conditions the process proved to be reversible according to the reheating criterion, and well represented by the two-state N ⇔ D transition model in the whole investigated pH range [13]. The thermodynamic parameters derived from the analysis of DSC curves are collected in Table 1. The data in Table 1 emphasize that there is a marked dependence of denaturation temperature on the solution pH: the value of T_d passes from 68.9°C at pH 2.0 to 93.2°C at pH 5.0, and to 77.8°C at pH 8.0. Therefore, in the pH range 2.0–5.0, the denaturation temperature increases by about 25°C, whereas, in the pH range 5.0–8.0, the value of T_d decreases by about 15°C. These data can be qualitatively accounted for by the Linderstrom-Lang model since the isoelectric point of tendamistat is 4.35 (i.e., it has 5 Asp, 4 Glu, 3 Arg, 1 Lys and 2 His). However, by deepening the thermodynamic analysis, a different picture emerges, as it is shown in the following.

The plot of $\Delta_d H(T_d)$ versus T_d proved to be linear [13]; the plot slope corresponds to the denaturation heat capacity change, $\Delta_d C_p$, and amounts to 2.9 kJ K^{-1} mol^{-1}. Since $\Delta_d H$ is a linear function of temperature and does not depend on pH for tendamistat, it is represented by the following relation:

$$\Delta_d H(T) = 110.5 + 2.9(T - 298.15) \text{ in kJ mol}^{-1}. \quad (1)$$

The temperature where $\Delta_d H$ vanishes, $T_H = 260$ K, is a universal temperature for tendamistat, because it is pH independent. The apparent pH dependence of $\Delta_d H(T_d)$ is the consequence of the variation in the

Table 1
Thermodynamic parameters of the thermal denaturation of tendamistat, obtained from DSC scans, at various pH values. Experimental values of T_d and $\Delta_d H(T_d)$ are obtained using Eqs. (15) and (18), respectively, in reference [13]; $\Delta_d S(T_d) = \Delta_d H(T_d)/T_d$. The values of Δn are calculated by means of Eq. (6). The values of $\Delta_d G(25^\circ C)$ are calculated by means of Eq. (7), using $\Delta_d C_p = 2.9$ kJ K^{-1} mol^{-1}; they are to be considered as true experimental data

pH	T_d (°C)	$\Delta_d H(T_d)$ (kJ mol^{-1})	$\Delta_d S(T_d)$ (kJ K^{-1} mol^{-1})	Δn	$\Delta_d G(25^\circ C)$ (kJ mol^{-1})
2.0	68.9	238	0.70	1.9	22.0
2.5	76.1	259	0.74	1.7	26.5
3.0	83.2	279	0.78	1.4	31.0
3.5	89.4	297	0.82	1.0	35.1
4.0	92.0	305	0.84	0.6	36.9
4.5	92.9	307	0.84	0.2	37.5
5.0	93.2	308	0.84	−0.2	37.7
5.5	91.3	303	0.83	−0.5	36.5
6.0	88.9	296	0.82	−0.7	34.9
6.5	85.9	287	0.80	−0.8	32.8
7.0	82.0	276	0.78	−0.7	30.3
7.5	79.4	268	0.76	−0.5	28.5
8.0	77.8	264	0.75	−0.1	27.6

T_d values on changing the solution pH. The standard denaturation Gibbs energy change, $\Delta_d G$, vanishes at T_d

$$\Delta_d G(T_d) = \Delta_d H(T_d) - T_d \Delta_d S(T_d) = 0. \quad (2)$$

This implies that the denaturation entropy change, $\Delta_d S$, depends upon the solution pH. In fact, by using the values of Table 1, it is represented, at pH 2.0, 5.0 and 8.0, respectively, by the following relations:

$$\Delta_d S(T, \text{pH}\,2.0) = 0.30 + 2.9 \ln (T/298.15) \text{ in kJ K}^{-1}\text{ mol}^{-1} \quad (3)$$

$$\Delta_d S(T, \text{pH}\,5.0) = 0.24 + 2.9 \ln (T/298.15) \text{ in kJ K}^{-1}\text{ mol}^{-1} \quad (4)$$

$$\Delta_d S(T, \text{pH}\,8.0) = 0.28 + 2.9 \ln (T/298.15) \text{ in kJ K}^{-1}\text{ mol}^{-1}. \quad (5)$$

Therefore, the temperature at which $\Delta_d S$ vanishes, T_S, changes on changing the solution pH. This thermodynamic analysis of DSC data for tendamistat leads to the conclusion that the variation in T_d on changing the solution pH is due to entropic effects [14]. Interestingly, theoretical studies by Dill and coworkers pointed out that the entropy of proton binding, rather than the charge energetics, makes up a significant part of the electrostatic Gibbs energy change associated with the unfolding process [15].

The pH dependence of the thermodynamic stability of tendamistat indicates that proton binding is coupled to the unfolding transition. The number of protons linked to the thermal unfolding in a two-state N ⇔ D transition can be calculated with the following relation [1]:

$$\Delta n = [\Delta_d H(T_d)/2.3\,R\,T_d^2]\,[dT_d/dpH]. \quad (6)$$

By applying Eq. (6) to the experimental data for the thermal denaturation of tendamistat, we obtain for Δn the values collected in the fifth column of Table 1. Clearly, the value of Δn depends upon the solution pH; the calculated values indicate that the protein binds two protons on unfolding at pH below 4.0, while above pH 5.0 approximately one proton is released.

In order to further characterize the pH dependence of tendamistat stability, we calculate the denaturation Gibbs energy change, $\Delta_d G$, by means of the well-known equation [1]

$$\Delta_d G(T) = \Delta_d H(T_d)\,[1 - (T/T_d)] + \Delta_d C_p (T - T_d - T \ln (T/T_d)) \quad (7)$$

which is exact in the assumption that $\Delta_d C_p$ is temperature independent. The calculated values of $\Delta_d G$ at 25°C for the different pHs are reported in column (6) of Table 1.

It is evident that, in the pH range 4.0–5.5, the stability is practically constant with $\Delta_d G$ around

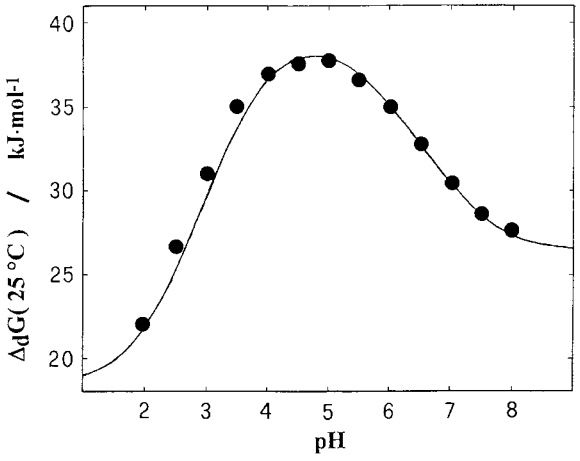

Fig. 1. Dependence of $\Delta_d G$ at 25°C on the solution pH for tendamistat. The solid circles are the experimental values reported in the sixth column of Table 1. The continuous curve represents the function calculated by means of Eqs. (10) and (11).

37 kJ mol^{-1}, whereas on lowering the pH from 4.0 and on increasing the pH from 5.5, the native structure becomes less stable: $\Delta_d G = 22.0$ kJ mol^{-1} at pH 2.0 and 27.6 kJ mol^{-1} at pH 8.0. The plot of $\Delta_d G$ versus pH at 25°C, that can be called the pH-dependent stability curve of tendamistat, is shown in Fig. 1: it resembles a bell-shaped curve, with two sigmoidal branches and a plateau in the pH range 4.0–5.5. Such shape may recall titration curves. However, Yang and Honig [16] emphasized that analyzing pH-dependent stability curves is fundamentally different from analyzing standard titration curves since the end-points rather than the mid-points serve as markes for the relevant pK_as. In fact, these authors, by means of a theoretical analysis and detailed calculations employing finite-difference numerical methods to solve the Poisson–Boltzmann equation [16,17], demonstrated that such a plot should not be interpreted in terms of groups whose pK_a corresponds to the titration mid-point, but rather in terms of groups with different pK_a values in the folded and unfolded states, corresponding, approximately, to the titration end-points. This is the key-point for further analysis and should be kept well in mind.

Lysine and arginine side-chains are in general well-exposed to solvent and, consequently, have pK_as that are essentially unshifted from their values in isolated amino acids. In contrast, the side-chains of several aspartic, glutamic and histidine residues possess anomalous pK_as in native proteins [16]. By coupling this general information to the key-point reported above, we can conclude that in tendamistat there should be two carboxyl groups whose pK_a values are around 4.0 in the unfolded state and below 3.0 in the native structure, and one histidine residue whose side-chain has a pK_a value below 6.0 in the unfolded state and above 7.0 in the native structure.

3. Thermodynamic model

By linking the proton binding to the conformational unfolding transition [18], we wish to develop a simple thermodynamic model, able to rationalize the experimental data. We consider a protein whose thermal unfolding is a two-state N ⇔ D transition, and we assume that both the native and denatured states have three sites for proton binding. The binding sites have different association constants in the two protein states. The linkage of proton binding to the thermal unfolding can be described by considering an overall equilibrium constant for the denaturation process, given by the sum of the concentrations of all of the unfolded species divided by the sum of all of the native species:

$$\mathbf{K} = K_u\left(\sum[DH_i]/\sum[NH_i]\right) = K_u(P_D/P_N), \tag{8}$$

where K_u is the equilibrium constant of the purely conformational transition N ⇔ D; and P_D and P_N are the binding polynomials [18] for the denatured and native state, respectively, in which the summation goes from $i = 1$ to 3 in order to take into account the concentrations of all the protonated species relative to the reference unprotonated species. Schellman pointed out that the binding polynomial is a macroscopic analog of the molecular semi-grand partition function, open to the ligand component for a fixed number of protein molecules [19]. The pH dependence of the denaturation process arises from the different values of the proton binding polynomials in the two protein states at various proton concentrations.

By applying a fundamental relation of equilibrium thermodynamics, the standard denaturation Gibbs

energy change is given by

$$\Delta_d G = -RT \ln \mathbf{K} = -RT \ln K_u - RT \ln(P_D/P_N) = \Delta_u G + \Delta\Delta_d G, \quad (9)$$

where the term $\Delta_u G$ represents the Gibbs energy change associated with the unfolding of native conformation, without considering variations in the ionization state of the protein (i.e., it includes contributions from conformational entropy, hydrophobic effect, H-bonds, packing interactions and desolvation of polar groups). The other term represents the contribution to the Gibbs energy change due to the binding of the three protons on ionizable groups.

The binding of such an ion as the proton to multiple sites in a single molecule is generally an anti-cooperative process because long-range electrostatic effects are operative. As a consequence, the binding polynomial, for both the native and unfolded states of tendamistat, can be factored into a product of individual-site terms, formally similar to that for heterogeneous independent binding sites [18]. Each individual-site term corresponds to a two-state system: occupied site and unoccupied site. Therefore, the term $\Delta\Delta_d G$ is given by

$$\Delta\Delta_d G = -RT \ln(P_D/P_N) = -RT \ln\{(1 + K_{b,D,1}[H^+])^2 \times (1 + K_{b,D,2}[H^+])/(1 + K_{b,N,1}[H^+])^2 \times (1 + K_{b,N,2}[H^+])\}, \quad (10)$$

where $[H^+]$ represents the molar concentration of protons; $K_{b,D,i}$ and $K_{b,N,i}$ are the association constants of the protons on the sites of denatured and native states, respectively. Clearly, in writing Eq. (10) we have considered that the two acid groups are identical both in the native and unfolded states; this assumption is physically reliable since they should be two carboxyl groups.

In general, the proton binding constant of an ionizable group is equal to the inverse of its acid dissociation constant: $K_b = 10^{pK_a}$. Furthermore, we assume that the pK_a values of the ionizable groups in the denatured state should correspond to those of the isolated amino acids. In fact, ionizable groups should be well-exposed to solvent in the denatured state, and charge–charge interactions should be negligible due to the screening effects of water molecules and counterions. In contrast, the pK_a values may be strongly different in the native state; local structural features and interactions contribute significantly in determining the observed pK_a values in the folded structure, rendering a challenging task the theoretical calculation of pK_a values to experimental accuracy [20,21].

We calculate the values of $\Delta\Delta_d G$ at 25°C, by means of Eq. (10), for different choices of the binding constants: (a) $K_{b,D,1} = 10^{3.9}\,M^{-1}$, $K_{b,N,1} = 10^{1.9}\,M^{-1}$, $K_{b,D,2} = 10^{5.8}\,M^{-1}$, and $K_{b,N,2} = 10^{7.5}\,M^{-1}$; (b) $K_{b,D,1} = 10^{3.9}\,M^{-1}$, $K_{b,N,1} = 10^{2.5}\,M^{-1}$, $K_{b,D,2} = 10^{5.8}\,M^{-1}$, and $K_{b,N,2} = 10^{7.6}\,M^{-1}$; (c) $K_{b,D,1} = 10^{3.8}\,M^{-1}$, $K_{b,N,1} = 10^{2.0}\,M^{-1}$, $K_{b,D,2} = 10^{5.4}\,M^{-1}$, and $K_{b,N,2} = 10^{7.6}\,M^{-1}$. The proton binding constant of the acid groups in the denatured state is selected taking in mind that pK_a is about 4.0 for a carboxyl group; while, the value of $K_{b,D,2}$ is selected by considering that pK_a is around 6.0 for His. Instead, the values of $K_{b,N,i}$ are selected on the basis of a visual analysis of the pH-dependent stability plot of tendamistat shown in Fig. 1. They, in fact, are related to the end of the two sigmoidal branches, at acid and basic pHs, respectively [16]. The three sets of calculated $\Delta\Delta_d G$ values are listed in columns 1–3 of Table 2. A quick glance at such values indicates unequivocally that the function $\Delta\Delta_d G$ depends strongly on the choice of pK_as.

A deeper analysis demonstrates that the choice (c) is the best one, because the $\Delta\Delta_d G$ values prove to be simply shifted downward by a constant quantity with respect to the experimental $\Delta_d G$ values, as it should be according to Eq. (9). Indeed, the quantity $\Delta_u G$ should be totally independent of pH by definition. The $\Delta\Delta_d G$ values calculated according to choice (c) are always positive except at pH 2.0 and 2.5; in particular, they are large and positive at pH 4.5–5.5. Such stabilizing contribution is in line with the results of detailed calculations showing that the electrostatic interactions between the charged groups in the native structure of tendamistat are optimized with respect to a random spatial distribution in the same structure [22]. The destabilization, due to the protonation of the two carboxylate groups in the unfolded state where p$K_a = 3.8$, is operative only at pH below 2.5; it continues as the solution pH is further lowered, until the two carboxylate groups become protonated also in the native state. This is a general mechanism, as well emphasized by Yang and Honig [16,17].

Table 2
Calculated denaturation Gibbs energy change of tendamistat at 25°C and different pH values

pH	$\Delta\Delta_dG$(25°C) (kJ mol^{-1})[a]			Δ_dG(25°C) (kJ mol^{-1})
	(1)	(2)	(3)	(4)
2.0	−9.2	−4.4	−4.6	21.9
2.5	−5.4	−2.5	−1.2	25.3
3.0	−0.8	0.8	3.2	29.7
3.5	3.6	4.5	7.2	33.7
4.0	6.8	7.5	10.1	36.6
4.5	8.5	9.1	11.4	37.9
5.0	9.0	9.6	11.4	37.9
5.5	8.6	9.2	10.5	37.0
6.0	7.4	7.9	8.6	35.1
6.5	5.5	6.0	6.3	32.8
7.0	3.4	3.8	3.9	30.4
7.5	1.7	2.0	2.0	28.5
8.0	0.7	0.8	0.8	27.3

[a] Note: values of column (1) are calculated by means of Eq. (10), using $K_{b,D,1} = 10^{3.9}$ M^{-1}, $K_{b,N,1} = 10^{1.9}$ M^{-1}, $K_{b,D,2} = 10^{5.8}$ M^{-1}, and $K_{b,N,2} = 10^{7.5}$ M^{-1}; values of column (2) are calculated by means of Eq. (10), using $K_{b,D,1} = 10^{3.9}$ M^{-1}, $K_{b,N,1} = 10^{2.5}$ M^{-1}, $K_{b,D,2} = 10^{5.8}$ M^{-1}, and $K_{b,N,2} = 10^{7.6}$ M^{-1}; values of column (3) are calculated by means of Eq. (10), using $K_{b,D,1} = 10^{3.8}$ M^{-1}, $K_{b,N,1} = 10^{2.0}$ M^{-1}, $K_{b,D,2} = 10^{5.4}$ M^{-1}, and $K_{b,N,2} = 10^{7.6}$ M^{-1}; values of column (4) are calculated by means of Eq. (11), $\Delta_dG = 26.5 + \Delta\Delta_dG$, using the $\Delta\Delta_dG$ values in column 3; see text for more details.

Following choice (c), the Δ_dG values at 25°C prove to be reproduced by the relation:

$$\Delta_dG = 26.5 + \Delta\Delta_dG, \quad (11)$$

where the $\Delta\Delta_dG$ values correspond to choice (c) and $\Delta_uG = 26.5$ kJ mol^{-1}. The values calculated with Eq. (11) are reported in column 4 of Table 2; a visual comparison between the calculated function and the experimental values, which are listed in column 6 of Table 1, is performed in Fig. 1. The agreement is good, confirming the strong relationship existing between the anomalous pK_a values and the pH-dependent stability curve of tendamistat.

It may appear strange that Δ_uG, the part of the denaturation Gibbs energy change devoid of any pH-dependent effects, amounts to only 26.5 kJ mol^{-1} at 25°C. Actually, this small number is the result of a significant cancellation between large but contrasting contributions to protein stability. Indeed, both the hydrophobic effect and the conformational entropy loss, which are the main factors in determining Δ_uG, amount to several hundreds of kilojoules per mole at room temperature, but they have opposite signs in the Gibbs energy balance. This is the origin of the characteristic marginal stability of globular proteins [1,4].

The ability of Eq. (11) to reproduce the pH dependence of Δ_dG indicates that tendamistat should possess two carboxyl groups with p$K_a = 2.0$ and one His side-chain with p$K_a = 7.6$ in the native structure. This means that in tendamistat there should be two local environments in which the pK_a values of two acid groups are substantially lowered with respect to the unfolded chain, and one local environment, where the pK_a of one His side-chain is substantially increased with respect to the unfolded chain. This can be explained with the existence of two salt-bridges involving the carboxyl group of two Asp or Glu residues, and the side-chain of one His and one Lys and/or Arg. Such salt-bridges, by considering that in the folded state p$K_a \approx 2.0$ for the two acid groups and p$K_a \approx 7.6$ and 10, respectively, for the basic groups, should be effective in stabilizing the native structure in a large pH range. On the other hand, the ionizable groups may not be involved in salt-bridges, and their anomalous pK_a values should be due to strong local interactions with H-bonding donors and/or acceptors that stabilize the charged form of the group.

The two histidines in tendamistat occur at positions 64 and 66, separated by Gly65 [10]. Analysis of the X-ray structure of the protein [10,12], Protein Data Bank

entry code 1hoe, reveals that the side-chain of His66 is well exposed to the solvent and is not involved in H-bonds with other residues; so, it should have a normal pK_a value. On the contrary, the side-chain of His64 is H-bonded to Oδ2 of Asp39 (Nδ1 of His64 is 2.87 Å away from Oδ2 of Asp39), which is also H-bonded to Thr41. Therefore, structural data suggest that His64 and Asp39 may have anomalous pK_a values and may be involved in a salt-bridge. In this regard, it should be noted that a salt-bridge is defined, according to several authors [23,24], as a pair of oppositely charged groups that form at least one H-bond with each other. Moreover, the side-chain of Glu38 is H-bonded to Nϵ of Arg68 and the peptide nitrogen of Glu38 is H-bonded to the carbonyl oxygen of His66. Thus, Glu38 may have a low pK_a value in the native structure of tendamistat.

To the best of our knowledge, the pK_as of the titratable groups of tendamistat have not been measured by NMR, hitherto. Therefore, even though the available structural information seems to confirm the results of our model calculations, this cannot be considered as a proof and caution is necessary. In any case, the devised approach indicates that the pH dependence of tendamistat thermodynamic stability can be rationalized by means of three ionizable groups, concentrated at a few spots on the protein surface, possessing anomalous pK_as. This view contrasts with the Linderstrom-Lang model, but reproduces the experimental data, and is validated by the theoretical analysis performed by Yang and Honig [16].

4. Discussion

Knowledge of the titration behavior of the ionizable groups determines the pH dependence of the protein thermodynamic stability [17]. Therefore, from a theoretical point of view, it is necessary to calculate the pK_a values of the ionizable groups in the protein from its native three-dimensional structure. This is a very complex problem for two reasons: (i) a protein with N ionizable residues has 2^N possible ionization states; (ii) the charged states of all the residues are coupled since they interact by long-range electrostatic forces. Some studies have used the finite-difference Poisson–Boltzmann method to calculate the pK_a values in native proteins, with reliable approximations for the statistical mechanical sums that describe the large number of possible ionization states of a protein [20,21]. Results of general validity have emerged. Anomalous pK_a values can arise in two different manners: (i) strong pair-wise charge–charge interactions, as in salt-bridges; (ii) strong interactions with nearby H-bonding donors and/or acceptors that stabilize the charged form of the group [16,17]. This leads to a simple interpretation of the pH dependence of globular protein thermodynamic stability. Consider a hypothetical protein having one acid and one basic residue which are so close by in the folded structure to form a salt-bridge, whereas they are so far away in the denatured state not to interact. If no other factors are operative, the pK_a value of the acid group will be smaller in the folded than in the unfolded state because the positive charge of the basic residue will stabilize the charged form of the acid. As a consequence, on lowering the solution pH, the conformational equilibrium will be shifted toward the unfolded state by mass action effects [16,17]. Bearing in mind these ideas, we have tried to rationalize the pH dependence of tendamistat thermodynamic stability emerging from experimental DSC data [13].

Such pH dependence can be rationalized by linking the binding of three protons on formally non-interacting sites, corresponding to three ionizable groups of the protein, to the conformational unfolding transition. Calculations indicate that the two acid groups linked to the thermal unfolding of tendamistat have p$K_a = 3.8$ in the unfolded state, consistent with solvent exposed carboxyl groups, whereas p$K_a = 2.0$ in the folded conformation, which is a very low value. Moreover, the third ionizable group of tendamistat should be a His side-chain with p$K_a = 5.4$ in the unfolded state and p$K_a = 7.6$ in the native structure. Actually, very low pK_a values for carboxyl groups have been determined in the native structure of several globular proteins. For instance, Asp66 in hen lysozyme has p$K_a = 0.9$ [16]; Asp94 and Asp102 in ribonuclease HI from *E.coli* have pK_as lower than 2.0 [25]; Asp93 in barnase has pK_a lower than 0.3 [26]; and Asp70 in T4 lysozyme has p$K_a = 0.5$ [9]. Similarly, in several proteins histidine residues show anomalous pK_as [7]; for instance, His30 in T4 lysozyme has p$K_a = 9.1$ [9]; and His40 and His92 in RNase T1 have p$K_a = 7.8$ and 7.7, respectively [27].

In conclusion, we have analyzed the pH dependence of the thermodynamic stability of tendamistat. A thermodynamic model, which links the proton binding to the thermal unfolding, is able to reproduce the dependence upon pH of the denaturation Gibbs energy function. The results indicate that tendamistat should have three ionizable groups with anomalous pK_a values in the folded structure.

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