SOLVENT ISOTOPE EFFECTS ON TAUTOMERIZATION EQUILIBRIA OF PAPAIN AND MODEL THIOLAMINES

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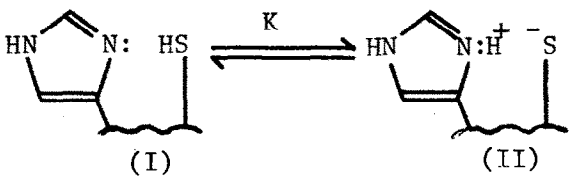
1. Introduction

The state of ionization of the active site sulfhydryl group of papain at the pH-optimum of activity (pH $\sim$ 6.5) remains a controversial issue. The proximity of an imidazole side chain to the sulfhydryl, established by chemical means [1] and from X-ray measurements [2], suggests the potential for a tautomerization equilibrium involving these two functions at this pH:



(1)

Polgar was the first to argue for the predominance of II in the active site based on the mercaptide ion-like ultraviolet difference absorption spectrum of papain versus carboxymethyl- and carboxamidomethylpapain near neutral pH [3]. Lewis et al. concluded that II is the predominant tautomer ($\sim$ 90%) based on one interpretation of the results of their titrimetric procedure [4]. On the other hand, all of the observations reported so far regarding the state of the tautomerization equilibrium are best viewed as being in a tentative stage of interpretation (discussion in [5]).

An alternative approach to this problem is reported here which utilizes the difference between the isotopic fractionation factor of the S–H bond ($\phi \simeq 0.4$ [6,7]) and the N–H bond ($\phi = 0.97$ [8]). In principle, the tautomerization equilibrium shown in eq. (1) should be subject to an inverse solvent deuterium isotope effect in which II is more stable relative to I in D_2O compared to H_2O ($K(H_2O)/K(D_2O) = \phi_{SH}/\phi_{NH} \simeq 0.4$). This principle has been successfully tested on the tautomerization equilibria of cysteine and β-mercaptoethylamine from spectrophotometric titrations. To the extent that these simple thiolamines model the spectral properties of the tautomeric species envisioned in the active site of papain, the tautomerization equilibrium constant for papain is estimated ($K(H_2O) \simeq 2$) from a comparison of the effect of solvent D_2O on the mercaptide ion spectra of the thiolamines and the mercaptide ion-like difference absorption spectrum of papain.

Abbreviation: L, hydrogen ion or deuterium ion

2. Materials and methods

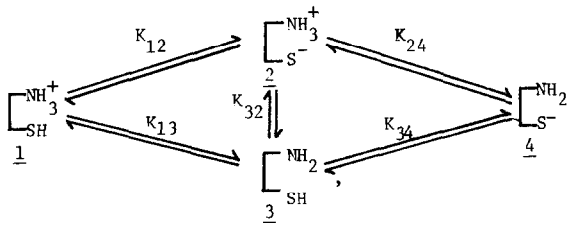
Papain (Worthington) was prepared by the method in [9]. L-Cysteine hydrochloride monohydrate (Sigma) was used without further purification. β-Mercaptoethylamine hydrochloride (Evans Chem.) was twice recrystallized from warm 1-propanol. Deuterium oxide, 99.8% (Bio-Rad), was distilled once under nitrogen. The sulfhydryl content of solutions of thiolamines was determined by an assay using 4-pyridine disulfide.

Absorption spectra of cysteine and β-mercaptoethylamine were determined as a function of pL on a Cary 219 Scanning Spectrophotometer. Difference spectra of papain versus its alkylated derivatives were taken on a Gilford 2400-2 spectrophotometer. Active

site concentrations in stock solutions of activated papain were determined with Ellman's reagent. For D_2O solutions, pD values were calculated as the pH meter reading plus 0.4.

3. Results and discussion

The general ionization scheme that accounts for the pH-dependent ionizations of small molecular weight thiolamines contains a tautomerization equilibrium formally analogous to that envisioned in the active site of papain, K_{32}:



In this regard, cysteine and β-mercaptoethylamine model two potential states of the tautomerization equilibrium in the active site in that for cysteine both 2 and 3 coexist in significant concentrations (K_{32} = ([2]/[3])≃2) while for β-mercaptoethylamine 2 substantially predominates over 3. These distributions were first calculated [10] from the variation of the mercaptide ion absorption spectrum of these thiolamines with pH on the assumption that the intrinsic extinction coefficient (ε) of the mercaptide ion of 2 is equal to that of 4 at their respective wavelengths of maximum absorbancy (λ_{max}) [10]. In support of this approach, very similar values of K_{32} have been determined for cysteine from (a) the pH-dependence of the Raman spectrum [11] and (b) a recent application of the Wegscheidler Principle using *S*-methylcysteine to estimate K_{13} [12].

In preparation for experiments on the enzyme, both cysteine and β-mercaptoethylamine were titrated in H_2O and D_2O by the spectrophotometric procedure [10] to determine (a) the effect of D_2O on the intrinsic spectral properties of the mercaptide ion containing forms 2 and 4 and (b) how a predicted inverse equilibrium solvent deuterium isotope effect on K_{32} would be reflected in the pL-dependent absorption spectra of these compounds. Figure 1 shows sample absorption spectra of cysteine at two different pL values in H_2O and D_2O. As demonstrated

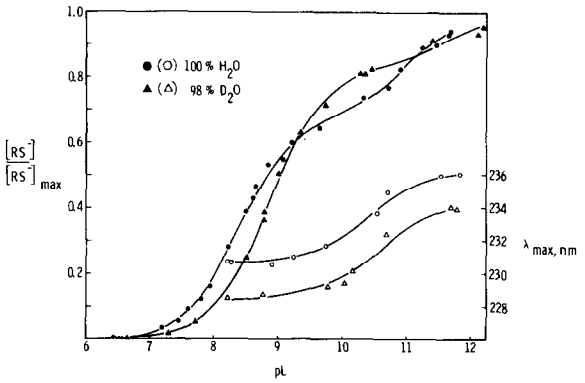


Fig.1. Ultraviolet absorption spectra of cysteine in 0.084 M NaOH (100% H_2O) (A) and 0.084 M NaOD (99% D_2O) (B); in wide range phosphate–borate–acetate buffer (16 mM in each buffer component), ionic strength = 0.084 M, at pH 10.25 (100% H_2O) (C) and pD 10.25 (99% D_2O) (D); and in 0.084 M HCl (100% H_2O) (E) and 0.084 M DCl (99% D_2O) (E).

[10] increasing pH produces both an increase in λ_{max} as well as an increase in the absorption at λ_{max} in H_2O; this also holds for D_2O solvent. There are two effects of D_2O on the spectra. At high pL (A,B), where form 4 is the exclusive species, D_2O produces a 2–2.5 nm blue shift in the spectrum due to a medium effect, although the ε at λ_{max} remains unchanged. At pL 10.25 (C,D), where cysteine exists as 32% 4 and 68% 2 plus 3 (based on the analysis in H_2O [10]), D_2O produces both a 2–2.5 nm blue shift in the spectrum, comparing λ_{max} (H_2O) with λ_{max} (D_2O), as well as an increase in the observed extinction coefficient at λ_{max}. This increase is attributed to an inverse equilibrium isotope effect on K_{32} as argued below.

Figure 2 summarizes the variation of obs. ε at λ_{max}, as well as the variation of λ_{max}, taken from absorption spectra of cysteine as a function of pL in H_2O and D_2O. The term $[RS^-]/[RS^-]_{max}$ was calculated as described in the figure and normalizes obs. ε against 1.0. As first argued [10], the first limb of the double sigmoidal curve is due to the conversion of 1 to 2 and its non-absorbing tautomer 3 while the second limb reflects the conversion of 2 and 3 to 4. The conversion of 2 to 4 in either solvent is also reflected in the increasing λ_{max} as pL increases. The λ_{max} for both 2 and 4 are subject to 2–2.5 nm blue shifts in D_2O. In contrast to cysteine, β-mercaptoethylamine exhibits nearly a single sigmoidal pL dependence due to the conversion of 1 to 2 and 3, fig.3. A second limb is only marginally detectable

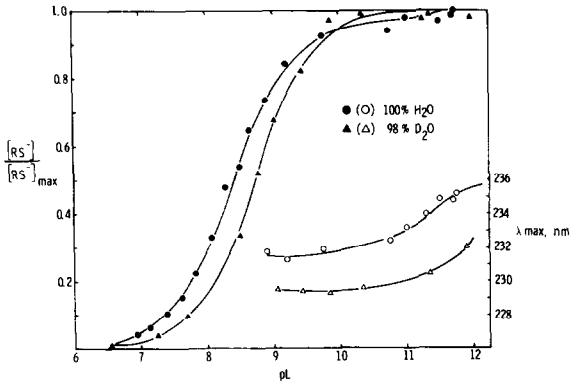


Fig.2. Spectrophotometric titration of cysteine in H_2O and D_2O in wide-range phosphate–borate–acetate buffers, 16 mM in each buffer component and ionic strength adjusted to 0.084 M using NaCl; [EDTA] = 0.5 mM; 25°C. Closed symbols reflect the variation of the term $[RS^-]/[RS^-]_{max}$ as a function of pL calculated from the equation $[RS^-]/[RS^-]_{max} = [(\epsilon_{RS^-}) - (\epsilon_{RSH})]/[(\epsilon_{RS^-})_{max} - (\epsilon_{RSH})_{max}$. The numerator is equal to the ϵ of cysteine at λ_{max} at a given pL minus the ϵ of cysteine in 0.1 N HCl (in H_2O) or 0.1 N DCl (in D_2O) at the same pL and the same wavelength. The denominator is equal the extinction coefficient of cysteine in 0.1 N NaOH (in H_2O) or 0.1 N NaOD (in D_2O) at λ_{max} $-\epsilon$ of cysteine in 0.1 N HCl (in H_2O) or 0.1 N DCl (in D_2O) at the same wavelength. Open symbols reflect variation in wave-length of maximum absorbance (λ_{max}) as a function of pL.

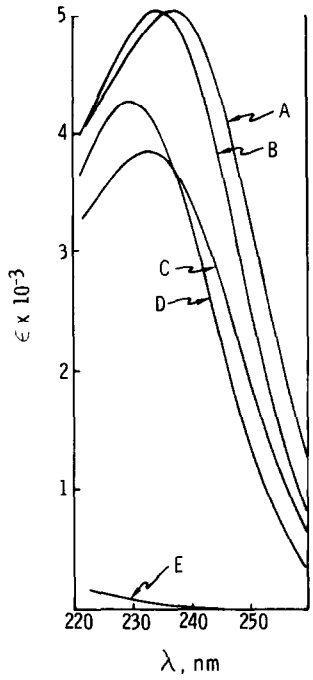


Fig.3. Spectrophotometric titration of β-mercaptoethylamine under the same conditions and using the same symbolism as in fig.1.

apparently because the equilibrium concentration of 2 far predominates over the non-absorbing tautomer, 3. However, the conversion of 2 and 3 to 4 above pL 10 is reflected in the increasing λ_{max} values. As for cysteine, the absorbance spectrum of 2 (pL <10) for β-mercaptoethylamine is subject to a 2–2.5 nm blue shift in D_2O. Form 4 is also subject to approximately the same blue shift based on the λ_{max} values for β-mercaptoethylamine in 0.1 N NaOH (in H_2O) and 0.1 N NaOD (in D_2O) which are 236–237 nm and 234–235 nm, respectively.

The titration curves shown in fig.2 and 3 could be adequately fitted by eq. (2) derived on the assumption that the intrinsic extinction coefficients of 2 and 4 are identical at their respective λ_{max} values in each of the two solvents:

$$\frac{[RS^-]}{[RS^-]_{max}} = \frac{K_{12}/K_{13} + K_{34}\,[L^+]}{[L^+]/K_{13} + K_{12}/K_{13} + K_{34}/[L^+] + 1} \qquad (2)$$

The computer best fit values of the microscopic ionization constants are shown in table 1. The value of the tautomerization equilibrium constant for cysteine in H_2O using the most accurate microscopic ionization constants ($K_{32} = K_{12}/K_{13} = 2.1$) corresponds to that in [10] within experimental error. In our hands, the microscopic ionization constants K_{24} and K_{34} were subject to large experimental error since their values depend on measuring small ΔA over a large background absorbance above pL 9.5, fig.2. The fact that the calculated solvent deuterium isotope effect on the tautomerization constant of cysteine (K_{32} $(H_2O)/K_{32}$ $(D_2O) = 0.41$) is in good agreement with theory ($K_{32}(H_2O)/K_{32}(D_2O) = \phi_{SH}/\phi_{NH} \simeq 0.4$) clearly supports the assumption on which eq. (2) was derived. An additional observation in support of nearly identical intrinsic ϵ of 2 and 4 at λ_{max} is that obs. ϵ at λ_{max} for β-mercaptoethylamine are nearly constant (≤5%) over pL 10.5–12.0 in both solvents, while the conversion of 2 to 4 is indicated from the increasing λ_{max} values. The isotope effects on sulfhydryl group ionizations (ΔpK_a) for cysteine and β-mercaptoethylamine, independent of the form in which they occur, are 0.3–0.4 while that for the

Table 1
Solvent deuterium isotope effects on the microscopic ionization constants for cysteine and β-mercaptoethylamine from spectrophotometric titrations[a]

Compound	Solvent	Ionization constants			
		pK_{12}	pK_{13}	pK^{b}_{24}	pK_{34}
Cysteine	D_2O	8.93 ± 0.05	9.63 ± 0.07	11.55 ± 0.03	10.85 ± 0.03
Cysteine	H_2O	8.56 ± 0.05	8.89 ± 0.07	10.99 ± 0.15	10.56 ± 0.15
	ΔpK_a	0.37 ± 0.1	0.74 ± 0.15	0.56 ± 0.45	0.29 ± 0.45
β-Mercapto-ethylamine	D_2O	8.74 ± 0.05			
β-Mercapto-ethylamine	H_2O	3.36 ± 0.05			
	ΔpK_a	0.38 ± 0.1			

[a] Ionization constants obtained from the computer best fit of eq. (2) to the data of fig.2,3
[b] Calculated from the best fit values to eq. (2) and the equality $K_{12}/K_{13} = K_{34}/K_{24}$

amino group ionizations are 0.5–0.7. These values compare reasonably well with solvent isotope effects on sulfhydryl group and amino group ionizations for compounds in which these groups do not occur in the same molecule [13].

Based on the results of the model studies, the magnitude of the tautomerization equilibrium constant of papain, eq. (1), was estimated from the relative effect of D_2O and H_2O on the ΔA spectrum of papain versus carboxymethyl- or carboxamidomethyl-papain in the 240–270 nm region (experimentally accessible because of the relatively low background absorbance of the protein) attributed to the long wavelength region of the absorption spectrum of an active site mercaptide ion [3]. The observations (fig.4) and arguments which lead to the conclusion of $K(H_2O) \simeq 2$ are as follows:

(1) The hypothesis that the ΔA at high pL is due to a mercaptide ion, is supported by two observations: (i) At high pL where the active site sulf-

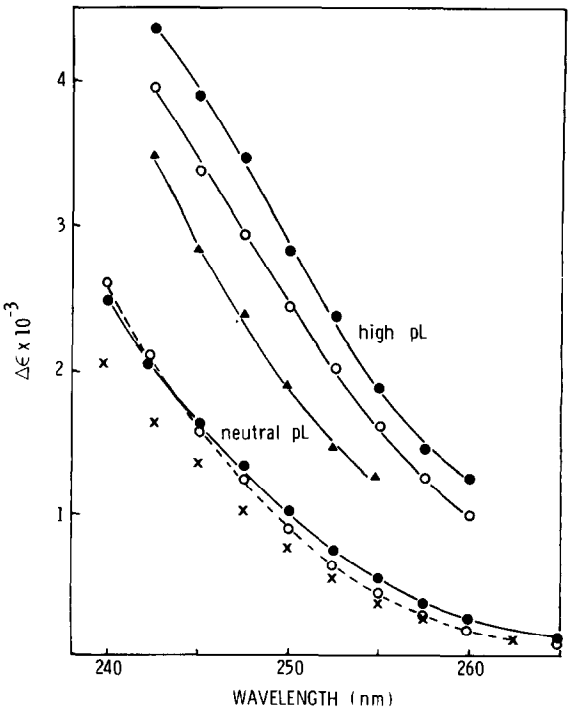


Fig.4. Ultraviolet difference spectra of papain (0.016 mM) versus carboxamido methylpapain (0.016 mM) at high pL in 100% H_2O (•, pH 10.0) and 98% D_2O (○, pD 10.26) and papain (0.03 mM) versus carboxymethylpapain (0.03 mM) near neutral pL in 100% H_2O (•, pH 6.5) and 98% D_2O (○, pD 6.5). The difference spectra were generated by the alkylation procedure detailed in [3]. The carboxamidomethyl derivative of papain was chosen for the high pL spectra because chloroacetamide reacts with papain at a conveniently fast rate, while chloroacetate reacts very slowly [14,15]. Conversely, the carboxymethyl derivative was chosen for the neutral pL spectra because chloroacetate reacts rapidly with the enzyme, while chloroacetamide reacts only slowly [14,15]. The ΔA is independent of whether chloroacetate or chloroacetamide is used in the alkylation procedure [3]. Theoretical spectra are shown due to: (a) a 2.5 nm blue shift of the observed spectra in H_2O near neutral pL (X); (b) a 2.5 nm blue shift of the observed spectra in H_2O at neutral pL plus a 25% increase in the extinction coefficients at all wavelengths (– – –); (c) a 5 nm blue shift of the high pL spectrum in H_2O (▲–▲).

hydryl is completely ionized, the difference spectrum is subject to a 2–2.5 nm blue shift in D_2O, an effect modeled by both cysteine and β-mercaptoethylamine at high pL, fig.1 (A,B), 2,3; (ii) The wavelength-dependent $\Delta\epsilon$ for the enzyme in H_2O and D_2O correspond to $\lesssim 10\%$ of the ϵ of cysteine at high pL in H_2O and D_2O, fig.1.

(2) >95% of the ΔA at pL 6.5 must be due to the tautomeric forms of papain, based on the demonstration that the appearance of the ΔA due to II is controlled by an app. $pK_a \simeq 4$ while the conversion of II and its non-absorbing tautomer III to the completely ionized form of papain at high pH is controlled by an app. $pK_a \simeq 8.5$ [3].

(3) The effect of D_2O on the difference spectrum of papain at pL 6.5 can be attributed to the combination of a 2.5 nm blue shift in the absorption spectrum due to II (X) plus a 25% increase in the app. ϵ of the blue-shifted spectrum at all wavelengths, resulting in a theoretical spectrum in D_2O (– – –) in close agreement with the observed spectrum in D_2O. This is qualitatively analogous to the effect of D_2O on the spectrum of cysteine at pL 10.25 in the 240–260 nm region where the tautomeric forms 2 and 3 coexist, fig.1C,D.

(4) Assuming that the intrinsic extinction coefficients of II in H_2O and D_2O are identical at pL 6.5, after correcting for the blue shift, the 25% increase in app. ϵ used to account for the spectrum in D_2O is interpreted as a 25% increase in II at the expense of the non-absorbing tautomer III. The assumption on which this conclusion is based is as good as the ability of cysteine to model the intrinsic spectral properties of the tautomeric forms of papain: The solvent isotope on K_{32} for cysteine was demonstrated to be in good agreement with theory based on an analysis in which the intrinsic ϵ at λ_{max} of 2 in H_2O and D_2O were assumed equal to the intrinsic ϵ 4 at λ_{max} in H_2O and D_2O, respectively. In so far as the intrinsic ϵ of 4 at λ_{max} are themselves equal in the two solvents (fig.1), the intrinsic extinction coefficients of 2 at λ_{max} in the two solvents are also equal.

(5) The tautomerization equilibrium constant for the enzyme in H_2O ($K(H_2O) = [II]/[I]$) can be calculated from eq. (3) in which $R = [II]_{D_2O}/[II]_{H_2O} = 1.25$:

$$K(H_2O) = \frac{R^{-}(\phi_{NH}/\phi_{SH})}{(\phi_{NH}/\phi_{SH})(1-R)} \quad (3)$$

Given the reported isotopic fractionation factors of the N–H bond ($\phi_{NH} = 0.97$ [8]) and the S–H bond ($\phi_{SH} \simeq 0.40$ [6,7]), $K(H_2O) \simeq 2$, corresponding to $\sim 66\%$ II in the active site.

(6) A similar value for the % II in the active site can be deducted independent of solvent isotope effects by assuming that the imidazolium ion of II causes a 5 nm blue shift in the absorbance spectrum of the neighboring mercaptide ion, in analogy with the 5 nm blue shift in H_2O of the mercaptide ion spectrum of form 2 of cysteine due to the neighboring ammonium ion, fig.2. A 5 nm blue shift of the observed spectrum at high pL for the enzyme in H_2O, fig.3, gives a spectrum that would be anticipated if the enzyme were 100% 2 near neutral pL (▲–▲). The difference between this spectrum and that observed near neutral pL in H_2O (●–●) could be accounted for by the presence of 40–50% non-absorbing III in the active site.

In summary, there are two observations reported here that may have an important bearing on the state of the tautomerization equilibrium in the active site of papain.

1. Solvent D_2O has been shown to be a predictable perturbant of tautomerization equilibria involving sulfhydryl groups, based on the results of the model studies.
2. There is a striking similarity between the effect of D_2O on the spectral properties of small molecular weight thiolamines and the mercaptide ion-like difference absorption spectrum of papain.

This has allowed an estimate of the tautomerization equilibrium constant for the enzyme based on the assumption that the thiolamines are good models of the intrinsic spectral properties of the tautomeric forms of the enzyme. On the other hand, the possibility that the spectral properties of papain are unique only to the enzyme and therefore a direct comparison with simple thiolamines inappropriate must still be considered. In this regard, a substantially larger tautomerization constant has been calculated for papain ($K(H_2O) = 8–12$) based on very different but no less arguable assumptions [4]. In any event, the particular experimental approach reported here must be given utmost consideration before any final deci-

sions are made with respect to the equilibrium distribution of tautomeric forms in the active site of papain.

Acknowledgements

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