SOLVENT ISOTOPE EFFECTS ON THE RATES OF ALKYLATION OF THIOLAMINE MODELS OF PAPAIN

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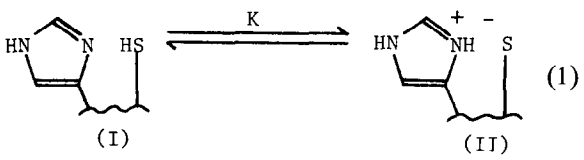
1. Introduction

Kinetic solvent deuterium isotope effects on the rates of alkylation of the active site sulfhydryl group of papain have been used as a probe of the mechanism by which this relatively simple nucleophilic process is catalyzed in the pH range where the enzyme is optimally active with substrates (pH $\simeq$ 5–7). As evidence against general base catalysis, unity isotope effects were reported on the rates of alkylation by methylbromoacetate and by chloroacetamide [1,2]. On the other hand, this interpretation is complicated by the reported inverse isotope effects on the rates of alkylation by bromoacetamide ($k(H_2O)/k(D_2O) = 0.74$) and by chloroacetate ($k(H_2O)/k(D_2O) = 0.75$) [1,3]. The possibility has been suggested that observed inverse kinetic isotope effects could arise from preferential reaction of these alkylating agents with the proposed active site thiolate–imidazolium ion pair, formed between the active site sulfhydryl group and a neighboring imidazole function, provided that the tautomerization equilibrium constant (K) controlling the relative concentrations of ion pair (II) and its tautomer (I) is ~2 in H_2O, eq. (1) [4]:



(I) (II) (1)

Thus, observed inverse kinetic isotope effects could originate from an equilibrium solvent deuterium isotope effect on K, estimated to be inverse from the isotopic fractionation factors of the S–H ($\phi \simeq 0.4$) and N–H ($\phi = 1.0$) bonds, $K(H_2O)/K(D_2O) = \phi_{SH}/\phi_{NH} \simeq 0.4$ [5].

As a test of whether it is chemically reasonable to attribute any or all of the observed kinetic solvent deuterium isotope effects reported on papain to the fundamental properties of the tautomerization equilibrium shown in eq. (1), the magnitudes of the solvent deuterium isotope effects on the rates of alkylation of the tautomeric forms of cysteine and β-mercaptoethylamine were determined for bromo- and chloroacetate, bromo- and chloroacetamide, as well as for methylbromoacetate. These thiolamines are viewed as elementary chemical models of the sulfhydryl group tautomerization equilibrium envisioned in the active site of papain.

2. Materials and methods

L-Cysteine hydrochloride monohydrate (Sigma) was used without further purification. β-Mercaptoethylamine hydrochloride (Evans Chemetics) was twice recrystallized from warm 1-propanol. Methylbromoacetate (Eastman) and bromoacetamide (Pfaltz) were used without further purification.

All kinetic measurements were made on a Gilford 2400-2 spectrophotometer equipped with a thermostatted cuvette carriage. The rates of reaction of alkylating agents with the thiolamines were determined as a function of pL in H_2O or D_2O from the rate of loss of mercaptide ion A_{250} in argon saturated phosphate–borate–acetate buffers, ionic strength adjusted with

Abbreviation: L, hydrogen ion or deuterium ion

NaCl. Pseudo-first order conditions were used in all cases. The observed pseudo-first order rate constants had to be corrected for a small first order loss of mercaptide ion absorbancy determined in the absence of alkylating agent. In the case of the brominated alkylating agents this correction was $\leq 2\%$ of the observed pseudo-first order rate constants, while for the chlorinated alkylating agents the correction was typically from 4–10% of the observed pseudo-first order rate constants.

Potentiometric titrations of the thiolamines were carried out on a TTT60 Titrator (Radiometer, Copenhagen) equipped with an ABU 12 Autoburette. Samples were maintained under argon in the presence of 0.1 mM EDTA during titration with NaOL in H_2O or D_2O. Ionic strength was maintained with 0.084 M NaCl.

All pD values were calculated as being equal to the pH-meter reading plus 0.4.

3. Results

The pL-dependence of the observed second order rate constants for reaction of alkylating agents with cysteine and β-mercaptoethylamine is described by eq. (2):

$$k^{obs} = \frac{k_1^{obs} + k_2 (K_B/[L^+])}{[L^+]/K_A + K_B/[L^+] + 1} \qquad (2)$$

derived from scheme (1) in which R = CO_2^- for cysteine and R = H for β-mercaptoethylamine; I = alkylating agent and P = alkylated thiolamine. The solvent deuterium isotope effect on k_1^{obs} is most important for comparison with the kinetic solvent deuterium isotope effects reported with papain since this is the second order rate constant for reaction with the sum of the tautomeric forms, 2 and 3. Fig.1 shows the experimentally determined pL-dependencies of k^{obs} using bromoacetate as an alkylating agent. The ascending limbs of the curves from pL ≃ 7–10 reflect the conversion of unreactive 1 to 2 plus 3, while the descending limbs of the curves (pL > 10) reflect the conversion of 2 plus 3 to 4. In order to simplify the

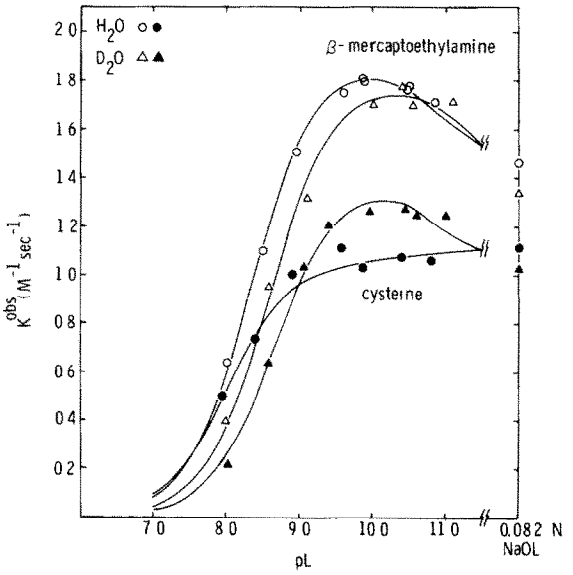
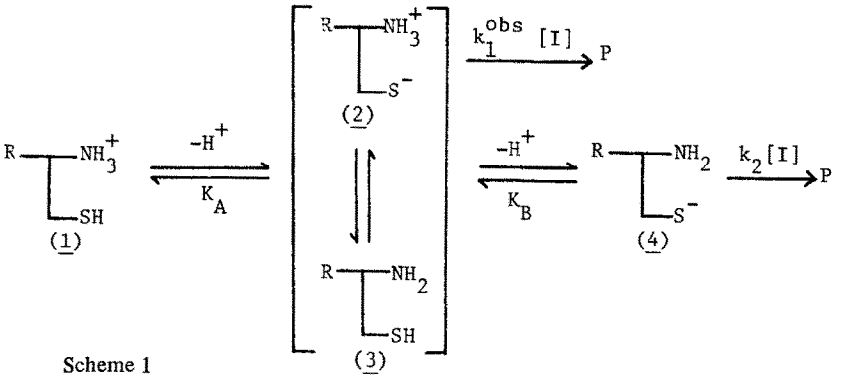


Fig.1. The pL dependence of the second order rate constants for alkylation of cysteine and β-mercaptoethylamine by bromoacetate in H_2O and D_2O (99%), 25°C. Pseudo-first order conditions were used for all determinations; cysteine and β-mercaptoethylamine were 0.1–0.2 mM while bromoacetate was 2–4 mM, depending on the pL. A wide range phosphate–borate–acetate buffer (16 mM in each buffer component) was used to maintain pL while NaCl was used to maintain ionic strength at 0.084 M.



Scheme 1

Table 1
Kinetically and potentiometrically determined macroscopic ionization constants for cysteine and β-mercaptoethylamine in H_2O and D_2O and the kinetic solvent deuterium isotope effects on the rates of alkylation of the pL-dependent forms of these thiolamines by bromoacetate

Parameter	Method	Cysteine in		β-Mercaptoethylamine in	
		H_2O	D_2O	H_2O	D_2O
pK_A	Potent. titr.[a]	8.10	8.59	8.34	8.68
pK_B	Potent. titr.[a]	10.49	10.89	10.84	11.38
$k_2(M^{-1}\ s^{-1})$	0.082 N NaOL[b]	1.11	1.03	1.47	1.34
$(k_2(H_2O)/k_2(D_2O))$		(1.08)		(1.10)	
pK_A	Curve fit[c]	8.03	8.66	8.34	8.58
$k_1^{obs}(M^{-1}\ s^{-1})$	Curve fit[c]	1.09	1.41	1.89	1.81
$(k_1(H_2O)/k_1(D_2O))$		(0.77)		(1.04)	

[a] Potentiometric titration of thiolamines in 0.084 M NaCl, 0.1 mM EDTA, 25°C. Na^+ correction applied above pL 10.50. Estimated error from repetitive titrations is ±0.05 pK units
[b] Determined from the time-dependent loss of mercaptide ion A_{250} under pseudo-first order conditions, [thiolamine] = 0.1 mM; [bromoacetate] = 2 mM in 0.082 N NaOH (in H_2O) and in 0.082 N NaOD (in D_2O). Ionic strength due to base plus bromoacetate is 0.084 M
[c] Best fit valves to the data of fig.1 using a least squares iterative computer program based on eq. (2) in which pK_B and k_2 were held fixed at the potentiometrically and kinetically determined values shown in the table

problem of determining k_1^{obs} by computer fitting eq. (2) to the data of fig.1, the following procedure was used:

(i) The macroscopic ionization constants for each of the thiolamines, pK_A and pK_B, were determined potentiometrically in H_2O and D_2O in the presence of 0.084 M NaCl and 0.1 mM EDTA, table 1.

(ii) The values of k_2 for reaction of bromoacetate with form 4 of the thiolamines were determined in strong base in H_2O and D_2O, table 1. The values of k_2 and K_B so determined were then held constant in a computer program based on eq. (2) which iterated about the values of K_A and k_1^{obs} until the least squares best fit values were found. The solid lines through the data of fig.1 are the least squares best fit lines obtained by this procedure; table 1 shows the best fit values for k_1^{obs} and K_A determined in H_2O and D_2O.

For bromoacetamide and methylbromoacetate complete pL-alkylation rate profiles could not be obtained because of the rapid rates of hydrolysis of these compounds above pL ≃ 9.5. Thus, the isotope effect on k_1^{obs} was calculated from k^{obs} values determined in H_2O and D_2O at relatively low pL ($K_B/[L^+] \ll 1$) where eq. (2) effectively reduces to eq. (3):

$$k^{obs} = \frac{k_1^{obs}}{[L^+]/K_A + 1} \qquad (3)$$

In practice, 3–5 repetitive measurements of k^{obs} were made in H_2O at pH = 8–8.4 and 3–5 repetitive measurements of k^{obs} were made in D_2O at pD = 8.2–8.6. To increase the comparative precision of these measurements, they were made on the same day using the same concentrated stock solution of alkylating agent dissolved in 50% H_2O/50% D_2O. At the stock concentrations of alkylating agent used, an insignificant 1% D_2O was introduced into the H_2O run and an insignificant 1% H_2O was introduced into the D_2O run. Finally, k_1^{obs} in each of the solvents was calculated using eq. (3) together with the kinetically determined values of K_A obtained from the computer fit to the complete alkylation rate profiles for cysteine and β-mercaptoethylamine by bromoacetate, table 1. Application of this procedure to bromoacetate gave values of k_1^{obs} in H_2O and in D_2O in close agreement with those determined from the curve fitting procedure, table 2. Table 2 also summarizes the solvent deuterium isotope effects on k_1^{obs}, and the corresponding standard deviations, determined by this method for bromoacetamide and methylbromoacetate as well as for chloroacetamide and chloroacetate.

Table 2
Solvent deuterium isotope effects on k_1^{obs} for cysteine and β-mercaptoethylamine with different alkylating agents[a]

Thiolamine	Alkylating agent	k_1^{obs}(M^{-1} s^{-1}) in		$k_1^{obs}(H_2O)$
		H_2O	D_2O	$k_1^{obs}(D_2O)$
Cysteine	bromoacetate[b]	1.03 ± 0.02	1.46 ± 0.03	0.70 ± 0.02
β-Merc.	bromoacetate[b]	1.97 ± 0.05	1.85 ± 0.08	1.06 ± 0.05
Cysteine	chloroacetate[c]	0.011 ± 0.001	0.013 ± 0.001	0.85 ± 0.10
β-Merc.	chloroacetate[c]	0.028 ± 0.002	0.030 ± 0.002	0.93 ± 0.07
Cysteine	bromoacetamide[b]	5.71 ± 0.10	8.34 ± 0.05	0.69 ± 0.01
β-Merc.	bromoacetamide[b]	8.79 ± 0.32	7.54 ± 0.28	1.17 ± 0.06
Cysteine	chloroacetamide[c]	0.061 ± 0.003	0.096 ± 0.003	0.64 ± 0.04
β-Merc.	chloroacetamide[c]	0.169 ± 0.006	0.157 ± 0.001	1.08 ± 0.04
Cysteine	methylbromoacetate[d]	25.8 ± 1.2	48.6 ± 1.1	0.53 ± 0.03
β-Merc.	methylbromoacetate[d]	42.6 ± 1.9	43.0 ± 2.5	0.99 ± 0.06

[a] Calculated with eq. (3) from 3–5 repetitive determinations of the observed second order rate constants in H_2O at pH ≃ 8–8.4 and 3–5 repetitive determinations in D_2O at pD 8.2–8.6, 25°C. The values of K_A used for the calculation in H_2O and D_2O were held constant at the kinetically determined values shown in table 1. A wide range phosphate–borate–acetate buffer (16 mM in each buffer component) was used to maintain pL while NaCl was used to maintain ionic strength at 0.084 M
[b] [Thiolamine] = 0.2 mM; [alkylating agent] = 4 mM
[c] [Thiolamine] = 1.0 mM; [alkylating agent] = 40 mM
[d] [Thiolamine] = 0.1 mM; [alkylating agent] = 2 mM

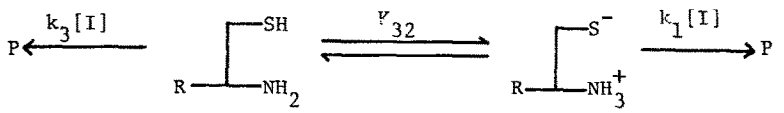
4. Discussion

Kinetic solvent deuterium isotope effects on the limiting observed rate constant for alkylation of the tautomeric forms of cysteine and β-mercaptoethylamine, k_1^{obs}, must be minimally determined by eq. (4) in which superscripts H and D refer to rate constants in H_2O and D_2O solvent:

$$\frac{k_1^{obs}(H_2O)}{k_1^{obs}(D_2O)} = \frac{k_3^H + k_1^H K_{32}^H}{k_3^D + k_1^D K_{32}^D}\left(\frac{1 + K_{32}^D}{1 + K_{32}^H}\right) \qquad (4)$$

The equation is derived from reaction scheme (2) in which I is alkylating agent, P is alkylated thiolamine, $K_{32} = [\underline{2}]/[\underline{3}]$ and R = CO_2^- and R = H for cysteine and β-mercaptoethylamine, respectively:

The isotope effects on k_1^{obs} for both cysteine and β-mercaptoethylamine with a variety of different alkylating agents are fully consistent with $\underline{2}$ being the reactive form towards alkylation ($k_1 \gg k_3$), table 2. In the case of cysteine, the isotope effects are uniformly inverse in the range 0.53 ± 0.03 to 0.85 ± 0.10 with rate constants that vary over 3 orders of magnitude. This range encompasses a predicted isotope effect of ~0.8 calculated from eq. (4) under conditions where $k_1 \gg k_3$, $k_1^H/k_1^D \simeq 1$ and given that $K_{32}^H \simeq 2$ and $K_{32}^D \simeq 5$, as estimated from the spectrophotometric titration of cysteine in H_2O and D_2O [4]. For β-mercaptoethylamine an inverse isotope effect on k_1^{obs} is not expected since $\underline{2}$ predominates over $\underline{3}$ such that $K_{32} \gg 1$ [4]. Under these conditions eq. (4) reduces to $k_1^{obs}(H_2O)/k_1^{obs}(D_2O) \simeq k_1^H/k_1^D$ when $k_1 \gg k_3$. Thus, the isotope effect on k_1^{obs} should only reflect



(3) (2)

Scheme 2

the intrinsic isotope effect on the rate of alkylation of the mercaptide ion containing form, 2. This isotope effect is anticipated to be near unity since no apparent proton transfers are involved in the alkylation of 2. The isotope effects on k_1^{obs} listed in table 2 for β-mercaptoethylamine are fully consistent with this expectation, $k_1^{obs}(H_2O)/k_1^{obs}(D_2O) = 0.93 \pm 0.07$ to 1.17 ± 0.06. Likewise, the isotope effects on the rates of alkylation of form 4 of cysteine and β-mercaptoethylamine are near unity, table 1. Finally, fig.1 graphically illustrates the basic kinetic differences between cysteine and β-mercaptoethylamine towards alkylation. For cysteine, in the range pL 9–11, the larger observed rate constants, k^{obs}, in D_2O compared to those in H_2O reflect the inverse equilibrium isotope effect on K_{32} in which 2 is the reactive form towards alkylation. No such inverse kinetic isotope effect is found for β-mercaptoethylamine since 2 already far predominates over 3 in H_2O ($K_{32} \gg 1$).

In view of the results of the model studies, the central question of interest is what explains the observed inverse and unity kinetic solvent deuterium isotope effects on the rates of alkylation of papain with these alkylating agents, table 3. On the one hand, the inverse isotope effects on k_1^{obs} for alkylation of papain by chloroacetate and bromoacetamide are modeled by the inverse isotope effects on k_1^{obs} for alkylation of cysteine by these alkylating agents. This supports the idea that the reactive form of the enzyme is the thiolate–imidazolium ion pair whose concentration is controlled by an inverse isotope effect on the tautomerization equilibrium constant, which in H_2O is ~2 as first suggested in [3,4]. On the other hand, the near unity isotope effects on k_1^{obs} for alkylation of papain by methylbromoacetate and chloroacetamide are modeled by the near unity isotope effects on k_1^{obs} for reaction of β-mercaptoethylamine with these alkylating agents and conforms to the active site conditions suggested [6] in which the tautomerization equilibrium constant for the enzyme is large, $K = 8–12$. The possibility has been suggested that observed unity isotope effects with the enzyme could in some cases be the result of a significant degree of reaction of the alkylating agent with the thiol–imidazole tautomer, thus, compensating for an inverse component due to an isotope effect on the tautomerization equilibrium constant of ~2 in H_2O [3]. While this possibility cannot be excluded, there is no clear evidence for any such effect for the alkylating agents tested with cysteine.

The primary difficulty with drawing conclusions based on direct comparisons between papain and the model thiolamines is that the isotope effects observed with the enzyme are almost surely under more complex kinetic control than the isotope effects observed with the thiolamines. This is indicated by two major differences between the thiolamine system and papain:

(1) The isotope effects of k_1^{obs} for the enzyme are neither uniformly inverse nor uniformly near one, table 3.

(2) The order of reactivities of the alkylating agents

Table 3

Comparisons between the kinetic solvent deuterium isotope effects on the rates of alkylation of the tautomeric forms of cysteine and of papain with various alkylating agents

Alkylating reagent	Limiting rate constant, k_1^{obs}(M^{-1} s^{-1}) for			
	Cysteine in		Papain in	
	H_2O	D_2O	H_2O	D_2O
Chloroacetate	0.011 ± 0.001	0.013 ± 0.001	2.35 ± 0.12	3.15 ± 0.12
$(k_1^{obs}(H_2O)/k_1^{obs}(D_2O))$	(0.85 ± 0.10)[a]		(0.75 ± 0.07)[b]	
Chloroacetamide	0.061 ± 0.003	0.096 ± 0.003	0.083	0.083
$(k_1^{obs}(H_2O)/k_1^{obs}(D_2O))$	(0.64 ± 0.04)[a]		(1.0)[c]	
Bromoacetamide	5.71 ± 0.10	8.34 ± 0.05	7.0	8.1
$(k_1^{obs}(H_2O)/k_1^{obs}(D_2O))$	(0.69 ± 0.01)[a]		(0.74)[d]	
Methylbromoacetate	25.8 ± 1.2	48.6 ± 1.1	3.5	3.5
$(k_1^{obs}(H_2O)/k_1^{obs}(D_2O))$	(0.53 ± 0.03)[a]		(1.0)[d]	

[a] This work: conditions as shown in table 2

[b] Conditions: phosphate–borate–acetate buffer (40 mM in each buffer component), ionic strength = 0.32 M, 25°C [3]

[c] Conditions: ionic strength = 0.07 M, 25°C [2]

[d] Conditions: ionic strength = 0.09 M, 25°C [1]

with the thiolamines are reversed in comparison with papain.

For example, k_1^{obs} for reaction of chloroacetate with papain is ~200-fold larger than k_1^{obs} for reaction of this alkylating agent cysteine, while k_1^{obs} for reaction of methylbromoacetate with papain is ~7-fold smaller than k_1^{obs} for reaction of this alkylating agent with cysteine, table 3. These differences might be understandable, if pre-equilibrium formation of an initial complex (C) between the ion pair form of papain (II) and alkylating agent were a central kinetic feature of the overall alkylation process:

$$I \overset{K}{\rightleftharpoons} II \underset{k_r}{\overset{k_f[I]}{\rightleftharpoons}} C \xrightarrow{k_1'} P$$

In this case, k_1^{obs}, is now a complex function of kinetic constants, eq. (5):

$$k_1^{obs} = \frac{k_f k_1'}{k_r + k_1'}\left(\frac{K}{K+1}\right) \quad (5)$$

Thus, the magnitude of the isotope effect on k_1^{obs} is controlled not only by the equilibrium isotope effect on K and its absolute magnitude but also by potential isotope effects on the remaining kinetic constants and their absolute magnitudes, eq. (6):

$$\frac{k_1^{obs}(H_2O)}{k_1^{obs}(D_2O)} = \left(\frac{k_f^H k_1'^H}{k_f^D k_1'^D}\right)\left(\frac{k_r^D + k_1'^D}{k_r^H + k_1'^H}\right)\left(\frac{K^H}{K^D}\right)\left(\frac{K^D+1}{K^H+1}\right) \quad (6)$$

Under only one condition does eq. (6) reduce to a relatively simple form; namely, when the rate constant for alkylation in the complex (C) is large compared to the dissociation rate constant ($k'_1 \gg k_r$), eq. (7):

$$\frac{k_1^{obs}(H_2O)}{k_1^{obs}(D_2O)} = \left(\frac{k_f^H}{k_f^D}\right)\left(\frac{K^H}{K^D}\right)\left(\frac{K^D+1}{K^H+1}\right) \quad (7)$$

This fact could provide an explanation for the lack of uniformity of the isotope effects on k_1^{obs} for the enzyme with different alkylating agents. For example, the isotope effect on k_1^{obs} for alkylation of papain by chloroacetamide has been reported to be unity while that for bromoacetamide is inverse, table 3. The observation that k_1^{obs} for bromoacetamide is nearly 100-times larger than that for chloroacetamide is most reasonably attributed to a much larger value of k_1' for the former alkylating agent, eq. (5). Thus, eq. (7) may apply in the case of alkylation of papain by bromoacetamide such that the observed inverse isotope effect on k_1^{obs} reflects inverse isotope effects on the binding rate constant (k_f) and/or the equilibrium terms of eq. (7). The near unity isotope effect on k_1^{obs} for the slower alkylating agent, chloroacetamide, would be comprehensible if eq. (6) describes the alkylation process such that a small normal isotope effect on k_1' and/or a small inverse isotope effect on k_r cancels the inverse contribution due to the equilibrium terms of eq. (6). By this reasoning, the observed inverse isotope effect on the alkylation rate of papain by chloroacetate would reflect $k_1' \gg k_r$ while the unity isotope effect with methylbromoacetate would reflect $k_1' \leq k_r$. This tentative conclusion seems reasonable in view of the possibility that electrostatic association between chloroacetate and the thiolate–imidazolium ion pair could be a primary factor in reducing the rate of dissociation from the enzyme.

In summary, the results of the model studies reported here emphasize the potential importance of inverse solvent deuterium isotope effects on the active site tautomerization equilibrium of papain as being the source of observed inverse kinetic solvent deuterium isotope effects found with some alkylating agents near neutral pL. However, it must be emphasized, that observed isotope effects with the enzyme are under relatively complex kinetic control compared to the model thiolamine systems, and therefore, alternative interpretations are possible.

Acknowledgments

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