Properties of methyl acetimidate and its use as a protein-modifying reagent

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The rate of hydrolysis of the imido ester methyl acetimidate and its rate of amidination of denatured aldolase were investigated under different conditions of temperature, pH and ionic strength. Both rate constants increase greatly with temperature, whereas ionic strength has no effect on either. The effect of pH is more complex. Between pH 6.8 and 8.8 the rate of hydrolysis decreases and the rate of amidination increases. These results are discussed in terms of the reaction mechanisms involved.

Methyl acetimidate, like all imido esters, modifies proteins by reacting with the ϵ -amino group of lysine residues (Hunter & Ludwig, 1962). It has a number of features that make it particularly useful as a reagent with which to probe protein structure. It is specific for lysine residues. As can be seen in Fig. 1, it is small, as is the adduct that it forms with lysine residues, and, furthermore, the positive charge of lysine is retained. It also reacts with lysine residues under very mild conditions (Hunter & Ludwig, 1962).

Some enzymes have been reported to suffer no significant loss in activity after amidination of several lysine residues by the reagent (Coggins, 1978). One enzyme, alcohol dehydrogenase, is even activated by methyl acetimidate (Browne & Kent, 1975b). It is therefore likely that it does not induce any gross conformational changes in these proteins.

It is readily hydrolysed in aqueous solution, but estimates of its half-life vary considerably. At pH 8.0 and 25°C, Hunter & Ludwig (1962) reported a value of 27 min. At pH 2.3 and 35°C, Coggins (1978) made an estimate of 17.5 min. By using the imido ester ethyl acetimidate, Browne & Kent (1975a) obtained values of approx. 5 min between pH 6.5 and 8.5 at 22°C. These latter authors used these findings to question the necessity of prolonged incubation of proteins with imido esters.

Preliminary experiments in our laboratory gave values for the half-life of methyl acetimidate in much better agreement with those of Hunter & Ludwig (1962) and Coggins (1978). Further, we showed that lowering the temperature during amidination of RNA polymerase led to an increase in the number of lysine residues modified (Makoff &

Malcolm, 1980). We also showed that raising the salt concentration led to an increase in modification of lysine residues of both RNA polymerase and aldolase (Makoff & Malcolm, 1980). We decided that an investigation into the effects of different reaction conditions on the rates of hydrolysis and amidination of this reagent was needed.

Materials and methods

Rabbit muscle aldolase was purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). Methyl [$1\text{-}^{14}\text{C}$]acetimidate was synthesized from [$1\text{-}^{14}\text{C}$]acetonitrile (The Radiochemical Centre, Amersham, Bucks., U.K.) by the method of Bates *et al.* (1975). Its specific radioactivity was determined as previously described (Makoff & Malcolm, 1980). The specific radioactivity was about 50 $\mu\text{Ci/mol}$, with individual batches varying by up to 10% from this value. Amidination in 30 mM-triethanolamine buffer and estimation of the number of lysine residues modified were carried out as previously described (Makoff & Malcolm, 1980).

In all experiments described in the present paper, aldolase was used in a denatured form by amidinating in 8 M-urea. In control experiments, all 26 lysine residues were modified by 100 mM-methyl acetimidate under these conditions.

Fig. 1. Reaction between the ϵ -amino group of lysine and methyl acetimidate

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Values quoted are obtained from least-squares straight lines calculated with a Commodore S-61 electronic calculator. 95% confidence limits (2 s.d.) are given where appropriate.

Results

The hydrolysis of methyl acetimidate was investigated under a variety of conditions of pH, temperature and ionic strength. The rate of hydrolysis was determined by incubating the reagent under the conditions of interest and estimating its concentration by measuring the number of lysine residues of denaturated aldolase it would modify. Such an estimation required calibration, which was performed by measuring the number of lysines modified at different concentrations of freshly prepared methyl acetimidate.

The effect of pH on the rate of hydrolysis was first investigated. Six pH values throughout the buffering range of triethanolamine buffer (6.8–8.8) were chosen and the rate of hydrolysis of methyl acetimidate was determined at each pH at 20°C and 0.1 M-NaCl. Calibrations of the number of

Fig. 2. Decay curve for methyl acetimidate at pH 8.0, 20°C and 0.1 M-NaCl

Methyl acetimidate (initially at 0.1 M) was incubated at 20°C in 0.2 M-triethanolamine buffer, pH 8.0, containing 0.1 M-NaCl. Samples were taken at various times and diluted 20-fold into a solution containing aldolase (0.3 mg/ml), triethanolamine (30 mM, pH 8.0), urea (8 M), NaCl (0.1 M), EDTA (0.75 mM) and dithiothreitol (0.75 mM). The number of lysine residues modified at each time point was determined and the methyl acetimidate concentration measured as described in the text.

lysine residues of denaturated aldolase modified as a function of reagent concentration were also carried out under the same conditions. A calibration curve for the experiment at pH 8.0 (results not shown) was used to construct the decay curve for the hydrolysis of methyl acetimidate shown in Fig. 2. If the hydrolysis is a first-order process, then $\ln[M] = k't$ (where $[M]$ is the methyl acetimidate concentration, t is the time and k' is the rate constant) and a plot of $\ln[M]$ against t should be a straight line with slope $-k'$. A semilogarithmic plot of the data from Fig. 2 gives a straight line ($r^2 = 0.998$) with a slope of -0.0207 ± 0.0017 , giving a value for k' at pH 8.0, 20°C and 0.1 M NaCl of $0.0207 \pm 0.0017 \text{ min}^{-1}$ and a half-life of 33 min.

Similar experiments were performed under the same conditions of temperature and salt concentration at different pH values. The rate constant and half-life were determined for each pH and these results are shown in Table 1. As expected, the rate constant for hydrolysis is pH-dependent, being approximately halved over a rise of 2.0 pH units.

The effect of salt concentration on the rate of hydrolysis was next investigated. For these experiments, methyl acetimidate was incubated at pH 8.0 and 20°C at different salt concentrations. The number of lysine residues of denaturated aldolase modified by the reagent after different incubation times was estimated as before, but in all cases at 0.1 M-NaCl. Because of this, only one calibration was performed, at pH 8.0, 20°C and 0.1 M-NaCl. The rate constants for hydrolysis and half-lives were determined as described above and are given in Table 2. As can be seen, there is no significant

Table 1. Effect of pH on rate of hydrolysis of methyl acetimidate at 20°C and 0.1 M-NaCl

pH	$10^{-2} \times k' \text{ (min}^{-1}\text{)}$	Half-life ($t_{1/2}$) (min)
6.8	2.42 ± 0.35	29
7.2	2.54 ± 0.12	27
7.6	2.30 ± 0.18	30
8.0	2.07 ± 0.17	33
8.4	1.59 ± 0.10	44
8.8	1.27 ± 0.05	55

Table 2. Effect of salt concentration on rate of hydrolysis of methyl acetimidate at 20°C and pH 8.0

[NaCl] (M)	$10^{-2} \times k \text{ (min}^{-1}\text{)}$	Half-life ($t_{1/2}$) (min)
0.1	1.92 ± 0.22	36
0.5	1.81 ± 0.21	38
1.0	1.85 ± 0.11	37
1.5	1.89 ± 0.14	37
2.0	1.85 ± 0.13	37

difference in the rate constants for hydrolysis of methyl acetimidate over a 20-fold range of NaCl concentrations.

The final variable to be investigated was temperature. Methyl acetimidate was incubated at 0.1 M-NaCl at different temperatures. For convenience, the pH (which was 8.0 at 20°C) was not adjusted for the effect of temperature on the pH of triethanolamine. (The temperature-dependence of the pH of triethanolamine is -0.02 pH unit/°C.) Because of this it was possible to estimate the number of lysine residues of denatured aldolase modified by methyl acetimidate at pH 8.0, 20°C and 0.1 M-NaCl after incubating the reagent at different temperatures. As with the salt-dependence experiments, only one calibration was therefore performed. The rate constants for hydrolysis and the half-lives were determined as before. They were corrected for the effects of pH by using the pH-dependence of k' given in Table 1. This correction was achieved by expressing k' as a percentage of its value at pH 8.0 and performing a linear regression. This best-fit straight line was used to convert the calculated values of k' to values at pH 8.0, which are given in Table 3. As expected, k' is strongly temperature-dependent, increasing by approx. 30-fold over a 40°C increase. An Arrhenius plot of the data in Table 3 gave a straight line ($r^2 = 0.9995$) with a slope of $-6980 \pm 410 \text{ K}^{-1}$, giving an activation enthalpy for the hydrolysis of methyl acetimidate of $58 \pm 3 \text{ kJ} \cdot \text{mol}^{-1}$.

The effect of these three variables on the rate of amidination of denatured aldolase was also examined. The calibration curves used in the determination of the pH-dependence of the rate of hydrolysis was also used to determine the pH-dependence of the rate constant for amidination (k). In a previous paper (Makoff & Malcolm, 1980) it was shown that, for a single class of lysine residues (L):

$$\ln \left(\frac{L}{L-L'} \right) = \frac{k}{k'} [M_0]$$

where L' is the number of lysine residues modified

and $[M_0]$ is the initial concentration of methyl acetimidate. For denatured aldolase, all 26 lysine residues should be chemically equivalent and so the above relationship should apply. Consequently, a plot of $\ln[L/(L-L')]$ against $[M_0]$ should be a straight line with a slope of k/k' . Such a semilogarithmic plot was performed for the data at pH 8.0, 20°C and 0.1 M-NaCl and is shown in Fig. 3. In this and other semilogarithmic plots there is some deviation from linearity at higher reagent concentrations. This is probably because at these concentrations the ratio of triethanolamine to methyl acetimidate is low. The decreased buffering capacity makes pH variation more likely. Because of this, the plots were analysed by performing linear regressions of the data up to 6 mM-methyl acetimidate. The plot in Fig. 3 has a slope of 0.0923 ± 0.0048 , which gives a value of $92.3 \pm 4.8 \text{ M}^{-1}$. The value of k can be calculated from this by using the value of k' at pH 8.0 determined above, giving $1.91 \text{ min}^{-1} \cdot \text{M}^{-1}$. The values of k were also determined at other pH points and are shown in Table 4. The pH-dependence of k is the opposite direction to that of k' , as an increase of 2.0 pH units leads to an

Fig. 3. Semilogarithmic plot of amidination of denatured aldolase by methyl acetimidate at pH 8.0, 20°C and 0.1 M-NaCl

Aldolase (0.3 mg/ml) was incubated overnight at 20°C with different concentrations of methyl acetimidate in 30 mM-triethanolamine buffer, pH 8.0, containing 8 M-urea, 0.1 M-NaCl, 0.75 mM-EDTA and 0.75 mM-dithiothreitol. The number of lysine residues modified at each reagent concentration was determined and the data plotted as $\ln[L/(L-L')]$ against $[M_0]$.

Table 3. Effect of temperature on rate of hydrolysis of methyl acetimidate at 0.1 M-NaCl

Temperature (°C)	pH	$10^{-2} \times k' \text{ (min}^{-1})$ (corrected to pH 8.0)	Half-life ($t_{1/2}$) (min) (corrected to pH 8.0)
0	8.4	0.317 ± 0.113	220
10	8.2	0.768 ± 0.050	90
20	8.0	1.92 ± 0.22	36
30	7.8	3.74 ± 0.29	19
40	7.6	8.52 ± 0.41	8.1

Table 4. *Effect of pH on rate of amidination of denatured aldolase at 20°C and 0.1 M-NaCl*

pH	k/k' (M^{-1})	k ($\text{min}^{-1} \cdot M^{-1}$)
6.8	34.5 ± 0.9	0.835
7.2	55.4 ± 6.4	1.41
7.6	72.4 ± 5.3	1.67
8.0	92.3 ± 4.8	1.91
8.4	126 ± 28	2.00
8.8	183 ± 99	2.32

Table 5. *Effect of salt concentration on rate of amidination of denatured aldolase at 20°C and pH 8.0*

[NaCl] (M)	k/k' (M^{-1})	k ($\text{min}^{-1} \cdot M^{-1}$)
0.1	97.7 ± 10.0	1.88
0.5	100 ± 26	1.81
1.0	93.7 ± 14.4	1.73
1.5	82.9 ± 15.8	1.57
2.0	89.8 ± 24.7	1.70

Table 6. *Effect of temperature on rate of amidination of denatured aldolase at 0.1 M-NaCl*

Temperature (°C)	pH	k/k' (M^{-1})	k ($\text{min}^{-1} \cdot M^{-1}$) (corrected to pH 8.0)
0	8.4	123 ± 22	0.293
10	8.2	124 ± 19	0.827
20	8.0	97.7 ± 10.0	1.88
30	7.8	88.9 ± 8.7	3.82
40	7.6	69.3 ± 8.6	7.82

approximate doubling of the rate constant in the pH range 6.8–8.8.

The effect of methyl acetimidate concentration on the number of lysine residues modified in denatured aldolase was also investigated at 20°C and pH 8.0, but at different salt concentrations. The value of k/k' and hence k were estimated in a similar fashion, and they are shown in Table 5. As with the rate constant for hydrolysis, the rate constant for amidination is independent of salt concentration over a 20-fold range of NaCl concentration.

The effect of temperature on the rate constant for amidination was examined in the same way. As with the experiments on hydrolysis of methyl acetimidate, the pH was at 8.0 (determined at 20°C) and not adjusted at other temperatures. Table 6 shows k/k' and k for each temperature. As with the temperature-dependence of the rate constant for hydrolysis, k was corrected to pH 8.0 by using the pH-dependence of the rate constant (in this case, Table 4). Like k' , k is strongly temperature-depen-

dent, a 40°C rise in temperature leading to a 27-fold increase in the rate constant. An Arrhenius plot of the data in Table 6 gives a straight line ($r^2 = 0.999$) with a slope of $-6940 \pm 600 \text{ K}^{-1}$, which gives an activation enthalpy for the amidination of aldolase of $58 \pm 6 \text{ kJ} \cdot \text{mol}^{-1}$.

Discussion

Our results clearly show that both the rate of hydrolysis and rate of amidination are strongly temperature-dependent. However, as the Arrhenius plots show, both rate constants have identical temperature-dependences. The increase in the number of lysine residues modified as a result of lowering the temperature was therefore due to the small pH change brought about by this temperature change.

In contrast with the effect of temperature, pH has quite different effects on the two rate constants. In fact, in the narrow pH range studied here, it acts in opposite directions. The effect of pH on k is the result of two opposing factors, since the reaction is believed to involve a nucleophilic attack by an unprotonated lysine ϵ -amino group on a protonated methyl acetimidate molecule (Hunter & Ludwig, 1962; Coggins, 1978). One would therefore predict a pH optimum between the pK_a for methyl acetimidate (approx. 7.5) and the pK_a for the ϵ -amino group of lysine (10.5). Our data probably only reflect the lower-pH side of this pH optimum.

When methyl acetimidate is hydrolysed, the protonated form may be attacked nucleophilically either by a neutral water molecule or by a negative hydroxyl ion (HO^-). If the former were more important, the rate of hydrolysis would increase with decreasing pH (increasing concentration of the cationic methyl acetimidate). If the latter were more significant, then the hydrolysis would decrease with pH, since $[\text{HO}^-]$ would be decreasing. In the pH range 6.8–8.8 the rate decreases, suggesting that most hydrolysis is performed by water rather than OH^- . This conclusion is supported by the lack of effect of ionic strength (Table 5). We cannot, of course, exclude the possibility that initial attack is by HO^- , but that subsequent H^+ -catalysed elimination of methanol is the rate-limiting step.

Salt concentration had no effect on either the rate of hydrolysis of methyl acetimidate or the rate of amidination of denatured aldolase. Yet both native RNA polymerase and native aldolase exhibit significant salt-dependence of the rate of amidination of their lysine molecules (Makoff & Malcolm, 1980). This presumably reflects the exposure of more lysine molecules of the native enzyme as a result of disruption of ionic interactions (such as salt bridges) by increased ionic strength.

Conclusion

Both hydrolysis and amidination are independent

of salt concentration and vary with temperature to the same extent over the range 0–40°C. In using methyl acetimidate (and presumably this applies to other aliphatic imidates) for protein modification, these parameters may be adjusted to suit other restrictions.

However, the pH variations are in opposite directions and therefore amidination should be carried out at as high a pH as is practicable within the range 6.8–8.8.

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