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REVERSIBLE INACTIVATION OF PAPAIN BY CYANATE*

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SUMMARY

Low concentrations of KCNO (approx. 0.1 mM) were found to react rapidly with activated papain (EC 3.4.4.10), leading to inactivation of the enzyme. The reaction proved to be reversible, the activity slowly returning again on sufficient dilution of the enzyme–KCNO mixture.

The reaction occurred with the essential thiol group of papain, shown by an amperometric method. The rate of inactivation could be conveniently determined from the shape of the progress curves of ester hydrolysis (5–120 mM benzoylarginine ethyl ester), traced on the recorder of a pH stat. The rate constant k was found to vary with substrate concentration. It decreased with increasing substrate concentration. A plot was made of k *versus* $[E]/([E] + [ES])$ as calculated from the known value of K_m. A linear relationship was found, which gave extrapolated values for $k = 9400\ \mathrm{M}^{-1}\cdot\mathrm{min}^{-1}$, at zero substrate concentration and of $k = 1500\ \mathrm{M}^{-1}\cdot\ \mathrm{min}^{-1}$ at infinite substrate concentration. The latter value shows that protection of the enzyme in the ES complex is not complete.

The rate constant of the reaction of the thiol group of free cysteine with KCNO under the same conditions (pH 6.0; 25°) was only $3.4\ \mathrm{M}^{-1}\cdot\mathrm{min}^{-1}$. Hence the reactivity of cyanate towards the thiol group of papain, as determined by extrapolation to zero substrate concentration, is 3000 times greater than the reactivity of the thiol group of free cysteine.

INTRODUCTION

When investigating the effect of urea on papain, HILL[1] made a rather paradoxical observation. On the one hand, the optical rotation of papain in 8 M urea was the same as in water and remained constant for at least 0.5 h, indicating that papain is stable to 8 M urea. This stability was later confirmed by optical rotatory dispersion measurements by the present author[2]. On the other hand, when papain was assayed in urea solutions a gradual decrease in activity to certain final levels

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Abbreviation: BAEE, benzoylarginine ethyl ester.

* A preliminary report on this work was presented at the First Annual Meeting of the Federation of European Biochemical Societies, London, March 1964.

of activity was observed. With increasing urea concentration the rate of inactivation increased and the final level of activity decreased. The assay experiments, therefore, seemed to indicate a gradual denaturation, One might conclude that denaturation occurs but is not accompanied by a change in the optical rotatory properties of the protein. Another explanation seemed feasible, however. STARK, STEIN AND MOORE[3] pointed out that urea solutions, on storage, contain low but increasing concentrations of ammonium cyanate, owing to the equilibrium of the well-known reaction:

$$NH_2\text{-}CO\text{-}NH_2 \rightleftarrows NH_4CNO$$

which is slowly attained when pure urea is dissolved. These authors showed that cyanate reacts rapidly with thiol groups and more slowly with amino groups. Since papain carries an essential thiol group, cyanate in the urea solutions could be the cause of the gradual inactivation of papain in 8 M urea under such assay conditions. This explanation may be inferred from the effect on papain of urea solutions of different age, as observed during the present investigation. Fig. 1 shows recorder traces of a pH stat during papain-catalyzed hydrolysis of 0.02 M benzoylarginine ethyl ester (BAEE) at pH 6.0, 25°. In the absence of urea the base *versus* time curve is nearly linear (Curve 1). In the presence of 6 M urea, dissolved immediately before the experiment, the conversion curve is curved downwards to a final slope which is a measure of the final, residual activity (Curve 2). This observation is similar to those of HILL[1].

When the experiments were carried out with urea solutions which had been stored at room temperature for 0.5 h and 2 h, the downward trend proved to be more marked and the final slopes lower (Curves 3 and 4). With a urea solution which had been stored overnight the reaction stopped almost immediately (Curve 5).

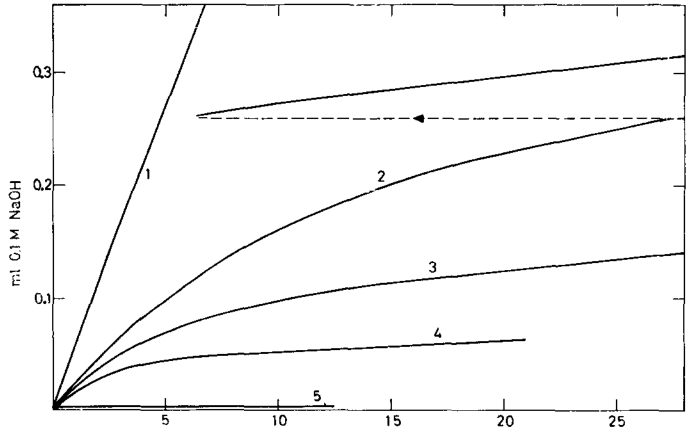

Fig. 1. The effect of urea solutions of different age on the progress curves of the hydrolysis of BAEE catalyzed by papain, as traced by the recorder of a pH stat. Conditions: 0.02 M BAEE, 6 M urea. Other conditions as indicated in EXPERIMENTAL. Curve 1, control in the absence of urea; Curve 2, reaction in freshly prepared urea solution; Curves 3, 4 and 5, reaction in urea solutions stored at room temperature for 0.5, 2 and 19 h, respectively. The difference in initial velocity between the control curve and Curves 2, 3 and 4 is due to 'competitive' inhibition exerted by urea[2].

Since, as mentioned above, cyanate is slowly formed in urea solution, the observed effects may be due to cyanate. The effect of cyanate on papain was therefore examined and was indeed found to inactivate papain and to show some interesting features.

EXPERIMENTAL

The pH stat equipment and the general procedures have been described in a previous paper[4]. The assay conditions for most of the experiments were 25°, pH 6.0, 0.3 M KCl and 5.0 mM cysteine *plus* 1.0 mM EDTA as activators. The addition of small aliquots to reaction mixtures was effected with syringes.

Papain was prepared by the method of KIMMEL AND SMITH[5]; BAEE was purchased from Calbiochem (Lucerne, Switzerland) and KCNO from Baker Chem. Co. (Phillipsburg N.J. "Baker Analyzed" reagent). In the experiments to be described (*cf.* Fig. 2) the BAEE concentrations were in the range of 5–120 mM, and the KCNO concentration in the range of 0.05–0.25 mM. Generally the lower KCNO concentrations were used in the presence of low substrate concentrations, the higher KCNO concentrations in the presence of high substrate concentrations. In this way progress curves were obtained with a low final slope (*cf.* Fig. 2, Curve 2) and a convenient reaction time for completion of the reaction.

Amperometric measurements were carried out in a titration vessel of 5 ml capacity. The agar salt bridge to the calomel electrode entered through the bottom of the vessel in order to save space at the top. The vibrating (50 Hz) Pt wire was of small size (0.25 mm × 3 mm) in order to limit the amount of Ag^+ consumed by the current during the experiment. The electrode served also as a stirrer. The 2 mM $AgNO_3$ solution was added from an "Agla" syringe (Burroughs-Wellcome Co., London) provided with a glass needle. The medium consisted of the imidazole buffer (pH 7.0) described in a previous paper[6]. Experiments were conducted at ambient temperatures (approx. 22°).

For the control reaction of Fig. 3, the papain was first incubated for 15 min in 7 ml of activator solution containing $8 \cdot 10^{-6}$ M KCNO, in the vessel of the pH stat, in order to approximately equilibrate the active papain with the dilute KCNO. The reaction was started by the addition of 3 ml of 0.1 M BAEE, making a total volume of 10 ml, $5 \cdot 10^{-6}$ M in KCNO and 0.3 M in KCl. For most of the experiments mercuri-papain was used, in order to avoid the short induction period which occurs if an aliquot of non-activated papain solution is added to a medium containing substrate, cysteine and EDTA. The mercuri-papain was prepared as follows. An aliquot of 15 ml of 1.5% papain, prepared according to KIMMEL AND SMITH[5], was 'dialyzed' by passing it through a 2.5 cm × 35 cm column of Sephadex G-25 superfine (Pharmacia, Sweden), using 0.02 M acetate buffer (pH 5.0) as eluant. Of the main papain peak eluted from this column 5 ml was made 5 mM in cysteine and 1 mM in EDTA. After 15 min at room temperature this aliquot was passed through a second (2 cm × 20 cm) column of Sephadex G-25, with a solution of 0.02 M acetate (pH 5.0) and 1 mM EDTA, as eluant. The main papain fractions were combined. Mercuric chloride was added, 1 mole of $HgCl_2$ per mole of papain, as determined by ultraviolet absorption ($E^{1\%}_{1\,\mathrm{cm}}$, 25). Thus mercury ions were actually in excess, since most preparations of papain had a thiol content of 0.4–0.6 SH per molecule. The EDTA does not prevent the mercury ions from combining with the papain thiol group[7].

Qualitative aspects

In order to examine the effect of cyanate the following procedure was adopted. In the reaction vessel of the pH stat the desired concentrations of substrate (BAEE), papain and activator were introduced in a total volume of 10 ml. After the hydrolytic reaction had proceeded for a few minutes, yielding a straight portion of the progress curve on the recorder, the desired amount of KCNO, dissolved in 0.1–0.2 ml of water, was added (Fig. 2). Gradual inactivation began as judged from the decreasing slope of the progress curves. The inactivation, however, did not proceed beyond a certain level, as can be seen from the nearly constant small slope of the final part of the progress curves. The incompleteness of the inactivation at these low KCNO concentrations may indicate that the inactivation reaction is a reversible one:

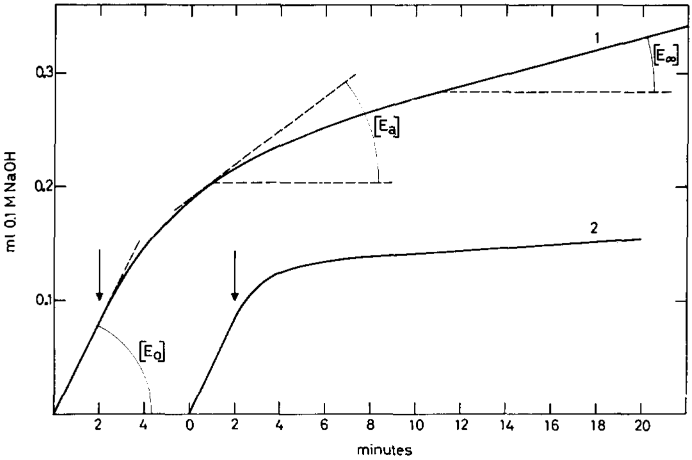

Fig. 2. Progress curves of the hydrolysis of 0.03 M BAEE previous to and after the addition of KCNO .The arrows indicate the moment at which KCNO was added (dissolved in 0.1 ml water). KCNO concentration in the reaction mixture $5.4 \cdot 10^{-5}$ M (Curve 1) and $17.9 \cdot 10^{-5}$ M (Curve 2).

$$E_a + C \underset{k'}{\overset{k}{\rightleftarrows}} E_i \qquad (1)$$

in which E_a indicates the active enzyme, C the cyanate and E_i the inactivated enzyme. In order to confirm this aspect, a stock solution of approx. $1.5 \cdot 10^{-5}$ M papain, containing 5 mM cysteine and 1 mM EDTA as activators, at pH 6.0, was made 0.17 mM in KCNO and stored at 22°. After 15 min a 0.3-ml aliquot of this solution was added to the reaction vessel of the pH stat which already contained substrate and activators so that the hydrolytic reaction could immediately begin. The result is shown in Fig. 3A, Curve 1. The activity, initially almost zero, returns slowly as judged from the increasing slope of the progress curve. This shows that the inactivation reaction is a reversible one.

During the reactivation reaction of Fig. 3A, Curve 1, the reaction mixture still contained a very low ($6 \cdot 10^{-6}$ M) concentration of KCNO, owing to the KCNO present in the 0.3-ml aliquot of papain added at the start of the reaction. $6 \cdot 10^{-6}$ M KCNO was therefore included in the control reaction (Fig. 3A, Curve 2). The final slopes of

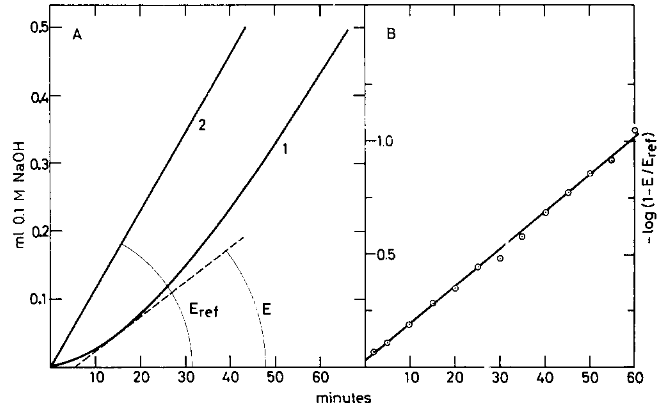

Fig. 3. A. Reactivation at pH 6.0, 25°, in 0.03 M BAEE, of papain previously inactivated by cyanate. Curve 1, reactivation; Curve 2, control (*cf.* EXPERIMENTAL). B. First-order plot of the reactivation.

Curves 1 and 2 are nearly the same, indicating complete reversibility of the inactivation reaction.

In another reactivation experiment a solution of cyanate-inactivated papain, similar to the one of the preceding experiment, was passed through a Sephadex column to remove the activator and the excess KCNO. To the combined papain containing eluant fractions mercuric chloride was added (*cf.* the preparation of mercuri-papain described in the experimental part) in order to protect liberated thiol groups. The solution was stored overnight in the refrigerator. The activity of the resulting papain was compared with the activity of papain treated in the same way, but omitting KCNO and was found to be virtually the same (95%). This again indicates complete reactivation.

Two pieces of data suggest that it is the thiol group which is involved in the inactivation reaction. It is evident from the data of Fig. 2 that the inactivation reaction is a fast one and STARK, STEIN AND MOORE[3] report that thiol groups are by far the most reactive towards KCNO. Secondly, pretreatment of non-activated papain (which carries no thiol group) with KCNO for 2 h had no effect on subsequent activity after activation*. Definite proof that the thiol group is involved was obtained by an amperometric method. To 3.0 ml of imidazole buffer (pH 7.0) small increments of 2 mM $AgNO_3$ were added, until a fair deflection of the galvanometer was obtained (Fig. 4, Curve a). This deflection was not quite stable but decreased slowly (Curve b). In the next experiment, after the blank titration, 0.4 ml of approx. 1% papain solution was added, which had previously been activated and freed from cysteine by passage through a Sephadex G-25 column (in 0.02 M phosphate buffer (pH 6.0)). The amount of papain was chosen such that the thiol content of the papain solution was slightly less than equivalent to the amount of $AgNO_3$ previously added to the titration vessel. Immediately upon addition of the activated papain solution the

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* At the start of the investigation a decrease of potential activity of non-activated papain was observed, which was fast, irreversible and only of limited extent (approx. 30%). Later (with other papain preparations) this could not be repeated despite many attempts to do so. The reason remains obscure. The reaction rate of the thiol group of papain was the same in both cases.

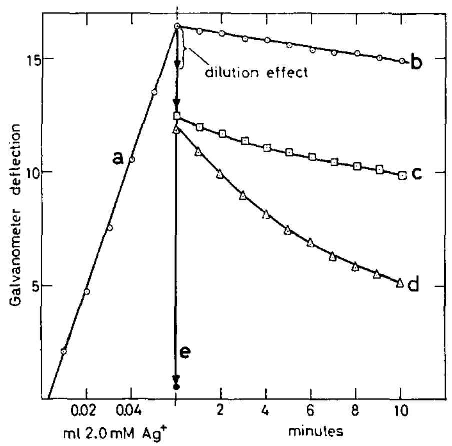

Fig. 4. Amperometric demonstration of absence and presence of thiol groups in papain preparations. Curve a, blank titration curve; Curve b, the slow drift of the galvanometer after the blank titration; Curve c, effect of adding 0.4 ml 1 % solution of non-activated papain; Curve d, effect of adding 0.4 ml 1 % solution of activated papain pretreated with cyanate; Curve e, effect of adding 0.4 ml 1 % solution of activated papain.

galvanometer deflection returned to almost zero, as expected (Fig. 4, Curve e). In a third experiment, the same amount of non-activated papain was added (Curve c). This also resulted in an immediate drop in the amperometric current, but only in a limited one. This drop was due partly to dilution of the Ag^+-buffer mixture with the added 0.4-ml aliquot, and partly to the presence of the protein, which seems to lower the diffusion current slightly*. In a fourth experiment the activated papain solution was made 15 mM in KCNO and stored for 15 min at room temperature in order to inactivate the papain. A 0.4-ml aliquot of this solution was then added to the titration vessel containing the $AgNO_3$ (Fig. 4, Curve d). The immediate, limited, drop in current was virtually equal to the drop observed in the experiment with the non-activated papain. Hence, in the cyanate-treated papain, the thiol group was initially absent. Thereafter silver ions were gradually consumed at a higher rate (Curve d) than in the control (Curve c), the thiol groups being slowly liberated again due to the inactivation reaction being a reversible one.

It may be concluded from this evidence that cyanate inactivates papain; that it does so by reaction with the essential thiol group, and that the reaction is a reversible one. These results are employed for the quantitative interpretation given in the next paragraph.

Quantitative aspects

According to Eqn. 1 the decrease in enzyme activity proceeds according to:

$$-\frac{d[E_a]}{dt} = k[E_a]\,[C] - k'[E_i] \qquad (2)$$

where k and k' denote the rate constants of the forward and reverse reactions,

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* According to our experience the ascending branch of an actual titration curve usually has a slightly smaller slope than a blank titration curve.

respectively, $[E_a]$ and $[E_i]$ denote the concentration of active and inactive enzyme and $[C]$ the concentration of KCNO. If $[E_0]$ is the initial concentration of active enzyme, proportional to the initial slope of the progress curve (Fig. 2), $[E_i] = [E_0] - [E_a]$. Substitution into Eqn. 2 yields

$$\frac{d[E_a]}{dt} = k'[E_o] - (k' + k[C])\,[E_a]$$

Since $[C] \gg [E_a]$ in all experiments, integration yields (*cf.* ref. 8):

$$\ln\frac{[E_a] - [E_\infty]}{[E_0] - [E_\infty]} = -\alpha t \tag{3}$$

where

$$\alpha = k' + k[C] \tag{4}$$

and

$$[E_\infty] = [E_o]\,k'/(k' + k[C]) \tag{5}$$

$[E_\infty]$ denotes the final activity, proportional to the final slope of the progress curve (Fig. 2).

The rate constants were derived by drawing tangents, usually at 0.25-min intervals, along the progress curves as indicated in Fig. 2, measuring the slopes $[E_a]$ and plotting $-\log\,([E_a] - [E_\infty])/([E_0] - [E_\infty])$ *versus* time. The slope of this plot equals $\alpha/2.3$.

Hence k and k' can be calculated from combining Eqns. 4 and 5:

$$k = \frac{\alpha}{[C]}\left(1 - \frac{[E_\infty]}{[E_0]}\right) \tag{6}$$

$$k' = \alpha\frac{[E_\infty]}{[E_0]} \tag{7}$$

Examples of such plots are given in Fig. 5. They show that Eqn. 3 is obeyed up to at least 90% of the total change in activity.

This method was used to study the effect of cyanate concentration on the rate of inactivation for a single substrate concentration (0.03 M BAEE). The data of two such experiments is given in Figs. 2 and 5. Experiments with two more cyanate concentrations were carried out and all results are given in Table I. The

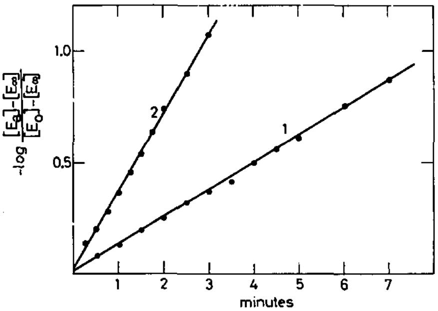

Fig. 5. First-order plots of the data of Fig. 2. Curves 1 and 2 correspond to Curves 1 and 2 of Fig. 2, respectively.

TABLE I

INDEPENDENCE OF k FROM CYANATE CONCENTRATION

Concn. $(M \times 10^4)$	k $(M^{-1} \cdot min^{-1} \times 10^{-3})$
0.54	4.4^8
1.12	4.4^4
1.79	4.3^6
2.24	4.4^3

k values calculated from the data are independent of the cyanate concentration, as expected.

From the final slope of the progress curve one can calculate the rate constant k' of the reverse reaction, using Eqn. 7. From Curve 1 of Fig. 2 a value of $k' = 0.035\ \text{min}^{-1}$ was obtained.

From the data of Fig. 3A the rate of reactivation can be directly determined. Fig. 3B shows a first-order plot of the return of the activity in Fig. 3A, the slope of the curve of the control reaction being used as the reference. The value obtained $k' = 0.037\ \text{min}^{-1}$ compares well with the value of 0.035 obtained from the data of Fig. 2, Curve 1.

The experiments were extended, using a number of different substrate concentrations in the range of 5–120 mM BAEE. The rate of inactivation was highly dependent upon substrate concentration. With increasing substrate concentration the rate of inactivation decreased, indicating protection of the thiol group, to cyanate, by substrate bound to the active site. This effect could be quantitatively explained by the following calculation.

In a reaction mixture the active papain molecules E_a are present either as free molecules E or as molecules of enzyme substrate complex ES. Therefore in the presence of cyanate the observed rate of inactivation k is actually the sum of the inactivation of free molecules and of molecules of ES complex:

$$k[E_a] = k_f[E] + k_c[ES]$$

in which k_f and k_c denote the inactivation rate constant of the free enzyme and of the enzyme–substrate complex, respectively. According to definition $[E_a] = [E] + [ES]$ and according to the Michaelis–Menten–Haldane treatment $[E] = [E_a]\,K_m/([S] + K_m)$. Substitution into Eqn. 8 yields

$$k = (k_f - k_c)\frac{K_m}{[S] + K_m} + k_c$$

Hence if k is plotted *versus* $K_m/([S] + K_m)$, a straight line should be obtained, which extrapolates to $k = k_f$ for $K_m/([S] + K_m) = 1$ (*i.e.* $[S] \rightarrow 0$) and to $k = k_c$ for $K_m/([S] + K_m) = 0$ (*i.e.* $[S] \rightarrow \infty$)*. Fig. 6 shows that such a plot is indeed linear. For K_m a value of 18 mM was used[9]. The extrapolations yield $k_f = 9.4 \cdot 10^3\ \text{M}^{-1} \cdot \text{min}^{-1}$ and $k_c = 1.5 \cdot 10^3\ \text{M}^{-1} \cdot \text{min}^{-1}$.

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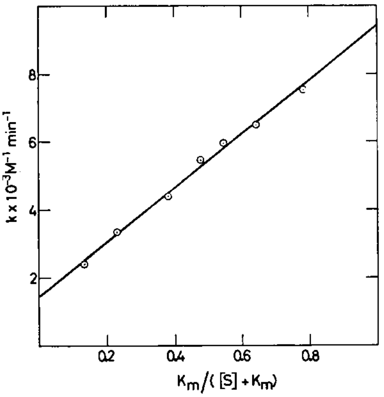
* It can be shown that $k = k_c\,k_3/(k_2 + k_3)$ for $[S] \rightarrow \infty$ in the more general case of enzyme action, in which the rates of acylation (k_2) and of deacylation (k_3) of the enzyme by the acid part of the substrate are taken into account. Since probably $k_2 \ll k_3$ for BAEE (see ref. 9) this case is not further considered here.



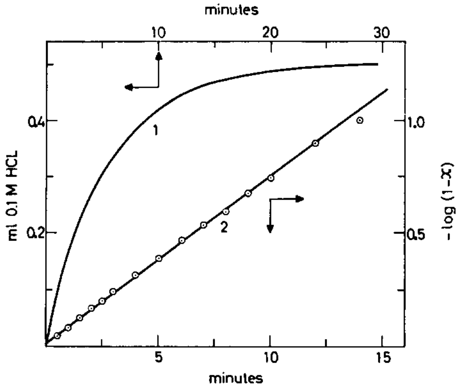


Fig. 6. Plot of inactivation rate k *versus* the fraction of active enzyme which is in the free state: $[E]/([E] + [ES]) = K_m/([S] + K_m)$ .The left-hand intercept equals the inactivation rate of the ES complex, the right-hand intercept the inactivation rate of the free enzyme. Each experimental point is the mean of 2–4 determinations, which mutually do not differ more than 7%.

Fig. 7. Curve 1, progress curve of the reaction of 5.0 mM cyanate with 50 mM cysteine at pH 6.0, 0.3 M KCl, 25°; Curve 2, first-order plot of the data of Curve 1.

It would be of interest to compare the reactivity of the thiol group of papain with the reactivity of the thiol group of free cysteine. The latter could be measured conveniently on the pH stat. Since cyanic acid is completely ionized at pH 6.0, the reaction proceeds according to:

$$\text{CySH} + \text{CNO}^- + \text{H}_2\text{O} \rightarrow \text{CyS–CONH}_2 + \text{OH}^-$$

The progress of the reaction could, therefore, be followed by automatic titration with 0.1 M HCl. An example of such a run is given in Fig. 7, Curve 1. Since cysteine (50 mM) was in excess of KCNO (5 mM) the reaction proceeded as a quasi first-order reaction. Hence the rate constant could be derived from the slope of the first-order plot (Fig. 7, Curve 2). The mean value of four such determinations, two having a lower (2 mM) KCNO concentration, gave a value of $3.4\ \text{M}^{-1}\cdot\text{min}^{-1}$, *i.e.* somewhat lower than the value of $4.0\ \text{M}^{-1}\cdot\text{min}^{-1}$ found by STARK, STEIN AND MOORE[3] using a chromatographic method of analysis. Hence the thiol group of free papain is nearly 3000 times more reactive than the thiol group of cysteine.

It was owing to this great difference in reactivity that experiments of the type shown in Fig. 2 could be carried out. The cysteine, at the low concentrations required for papain activation, did not greatly diminish the KCNO concentrations during the runs.

DISCUSSION

As far as the initial purpose of the investigation is concerned, the data presented adequately explain the behaviour of active papain in urea solutions. The gradual loss of activity is due to chemical modification of the essential thiol group and not to a slow change of conformation of the enzyme.

In view of the known reaction of cyanate with thiol compounds[3] it seems likely that the inactivation of papain takes place according to the overall reaction:

$$\text{P–S–H} + \text{HCNO} \rightarrow \text{P–S–CONH}_2$$

Whether the reactivation reaction is exactly the reverse one[3] or proceeds according to:

$$\text{P–S–CONH}_2 + \text{H}_2\text{O} \rightarrow \text{P–S–H} + \text{CO}_2 + \text{NH}_3$$

remains to be elucidated. In the latter case cyanate could be considered as a very bad substrate.

It has been found (Fig. 6) that, with increasing substrate concentration, the rate of inactivation of papain by KCNO decreases, which indicates that substrate retards the reaction. On the other hand HOLLAWAY, MATHIAS AND RABIN[10] observed that the rates of reaction of the related thiol enzyme ficin with chloroacetamide and iodoacetamide were accelerated in the presence of (a single concentration of) substrates, when compared with the rate in the absence of substrate. This suggests an effect opposite to the effect now found with papain and cyanate. Unfortunately it was not possible to determine accurately the rate of inactivation of papain with cyanate in the absence of substrate, owing to the high rate of inactivation and the reversibility of the reaction.

Two features emerged from this investigation which merit some comment.

(1) In the *ES* complex protection of the thiol group of papain against the action of cyanate is not complete; some 16% of the reactivity is retained. This is similar to a previous observation[9]: in the complex of substrate and non-activated papain the rate of activation by cysteine is around 7% of the rate of activation of free non-activated papain. Both observations indicate that the thiol group is located at the periphery of the substrate in the *ES* complex. The different extent of protection in the two cases may be due to the different size of the cyanate and cysteine molecules.

In a recent paper a partial protection of creatine kinase against inactivation by iodoacetic acid in the *ES* complex has been reported[11]. The latter enzyme, however, carries two thiol groups, which fact may make the cases of papain and creatine kinase not quite comparable.

(2) The thiol group of papain is considerably more reactive toward cyanate (about 3000 times) than is the thiol group of free cysteine. Such high reactivity occurs not only with cyanate but was also observed with bromoacetic acid: in one preliminary experiment in the presence of 0.013 M BAEE at pH 6.0, 40°, bromoacetic acid was found to react 15 000 times faster with papain than with cysteine.

Unusually high reactivity of protein groups in active sites of enzymes has been observed before. At pH 5.0 one of the imidazole groups in the active centre of ribonuclease is 500 times more reactive towards iodoacetic acid than is the imidazole group of acetyl histidine[12]. The thiol group of ficin has a reactivity towards chloroacetamide which is approx. 20 times that of most simple thiol compounds when the p*K* values of the thiol groups are taken into account[10]. Thus it would seem that in the case of papain the high reactivity of the essential group involved is more marked than in the other cases. However, since the p*K* value of the thiol group of papain is as yet unknown, an interpretation requires more data.

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