A NOVEL REACTIVITY OF PAPAIN AND A CONVENIENT ACTIVE SITE TITRATION IN THE PRESENCE OF OTHER THIOLS

K.BROCKLEHURST and G.LITTLE

Department of Biochemistry and Chemistry, Medical College of St. Bartholomew's Hospital, Charterhouse Square, London, E.C.1, England

Received 12 June 1970
Revised version received 17 June 1970

1. Introduction

Bender et al. [1] have discussed the methods available for the determination of the absolute concentrations of active sites of hydrolytic enzymes and have convincingly demonstrated the preference for a stoichiometric titration rather than for an assay based on measurement of rates of catalysis. The reagent chosen by these authors for the titration of papain is *p*-nitrophenyl *N*-benzyloxycarbonyl-L-tyrosinate (NBT) which does not satisfy all the requirements for an optimal titrant and has several disadvantages. Many of these are associated with the fact that it is a "specific" substrate for papain and hence the intermediate formed by acylation of papain by NBT undergoes rapid deacylation. Another type of titrant for thiol-enzymes is a reagent which reacts stoichiometrically with the enzyme to give a stable inactive derivative, i.e. an irreversible inhibitor. The use of an inhibitor rather than a substrate obviates the difficulties in the titration associated with the rapid turnover reaction. Many such reagents have been used to titrate thiol enzymes [e.g. see 2–6]. The chief disadvantage in using this type of reagent is that the reaction usually depends upon uncomplicated chemical reaction of the active site thiolate ion of the enzyme with the reagent and will not distinguish easily the thiol group in an intact active site from those in protein fragments, denatured enzyme, or other thiol contaminants such as activators. This difficulty could be obviated by using an active site directed inhibitor [7] which hopefully would react much more rapidly with the thiolate ion of the enzyme than with contaminant thiols. Active site directed inhibitors for papain which give easily followable reactions are not readily available, however.

This paper reports an unusual reaction of papain with 2,2′-dipyridyldisulphide (2PDS) the rate of which is optimal at pH 3.75 and which in acidic media is very much faster than the reaction of 2PDS with acid-denatured papain or with L-cysteine or 2-mercaptoethanol.

2. Materials and methods

Papain was obtained from three sources (1) B.D.H. (2 × crystallized) (2) Worthington (2 × crystallized) (3) the 3 × crystallized product of papain purified from B.D.H. papaya latex by the method of Kimmel and Smith [8]. 2PDS and 4PDS were obtained from Aldrich. Papain was activated with dithiothreitol and the activator removed by passage through a Sephadex G25 column. Deoxygenated deionized water containing 10^{-3} M EDTA was used throughout.

The kinetics and stoichiometries of the reactions of 2PDS with papain, L-cysteine and 2-mercaptoethanol were determined as follows. In the sample compartment of a Cary 15 or Cary 16K Spectrophotometer was placed a cell containing buffer (1.0 ml) and 10^{-3} M EDTA solution ([1.9–x] ml – see below). The absorbance of this cell at 343 nm was balanced against a cell in the reference compartment of the spectrophotometer containing buffer (1.0 ml) and 10^{-3} M EDTA solution (1.9 ml). To the sample cell was added a small volume (x ml, usually ca. 0.02–0.25 ml) of the thiol or enzyme solution and in the case of enzyme

solutions, the resulting small increase in absorbance recorded. The reaction was started by the addition of a solution of 2PDS in water (0.1 ml of 1.5×10^{-4} M or 1.5×10^{-3} M) first to the reference cell and then to the sample cell whilst the recorder was switched on. The addition of 2PDS solution to the sample cell was made as the pen crossed a marked line on the chart. The absorbance change in the reaction is given by final absorbance – (initial absorbance × 19/30). The correction to the initial absorbance for the low initial absorbance observed in this work may be neglected. The stoichiometry of the reaction was calculated using $\epsilon_{343} = 7.06 \times 10^3$ for the product 2-thiopyridone [9].

3. Results and discussion

The use of 2PDS as a reagent for thiols was first investigated by Grassetti and Murray [9] but the kinetics of these reactions were not reported. Second order rate constants for the reactions of 2PDS with papain, L-cysteine and 2-mercaptoethanol at pH values ca. 2.5, 4, 6 and 8 are presented in the table. The rate of the unusually fast reaction of papain with 2PDS in the pH range 2.5–5.0 is described by the equation

$$k = \bar{k}/(1 + [H^+]/K_1 + K_2/[H^+]) \text{ where}$$

$\bar{k} = 5 \times 10^4$ M^{-1} sec^{-1} and $pK_1 = pK_2 = 3.75$ (from the analysis of 25 experimental points). In the pH range ca. 2.5–9.0 all the reactions of 2PDS both with papain and with the simple thiols went to completion, the stoichiometry corresponding to the calculated concentrations in the case of simple thiols and to ca. 0.4–0.6 thiol/mole for papain. Similar results were obtained using papain from the three sources referred to in the Materials and methods section. The thiol content of the papain as determined by titration with 2PDS at all pH values corresponded closely with that determined by titration with DTNB at pH 8.0 and with that determined by titration with NBT at pH 3.2. That the same stoichiometry, that corresponding to the enzyme thiol as determined by other methods, is observed at all pH values in the reaction of papain with 2PDS suggests that the same group, probably the enzyme's only thiol group which is in the side chain of the active site cysteine residue (residue 25) [10, 11] is the reaction site for 2PDS at all pH values. Although

Table

Second order rate constants[a] for the reactions of 2PDS with active papain, L-cysteine and 2-mercaptoethanol at 25.0° and $I = 0.1$.

Thiol	pH	10^3 k, M^{-1} sec^{-1}
Papain	2.30[b]	4.58
	3.80[b]	17.10
	6.28[c]	0.90
	8.05[d]	1.14
L-Cysteine	3.90[b]	0.13
	6.25[c]	1.29
	8.00[d]	9.29
2-Mercaptoethanol	4.58[e]	0.014
	6.60[c]	0.105
	8.10[d]	4.25

[a] Second order rate constants were obtained either from conventional second order plots with [2PDS] ⩾ 2[thiol or enzyme] or when [2PDS] ⩾ [thiol or enzyme] by dividing pseudo first order rate constants by [2PDS]; in some cases both methods were used and concordant results obtained.
Buffers: [b]formate, [c]phosphate, [d]tris, [e]acetate.

what is presumably a nucleophilic attack on 2PDS which is much faster at pH 3.8 than at pH 8.0 (see table) is completely uncharacteristic of a thiol group, the view that this is indeed the reactive centre in the enzyme is compelled by the stoichiometry of the reactions at different pH values together with the following evidence. Papain which had been allowed to react with an excess of 2PDS at pH 3.8 and separated from the excess 2PDS on a Sephadex G25 column, failed to react either with 2PDS at pH 7.6 or with DTNB at pH 8.0. A control sample of enzyme which had been allowed to stand at pH 3.8 for an equivalent period of time followed by passage through a Sephadex G25 column was found to contain the expected thiol content as determined by titration with 2PDS and with DTNB. Papain blocked with 2PDS at pH 3.8 and separated from the excess reagent was completely inactive towards α-*N*-benzoyl-L-arginine ethyl ester (BAEE) at pH 6.5. Treatment of the 2PDS-blocked papain with an excess of 2-mercaptoethanol at pH 8.0 both released the expected quantity of 2-thiopyridone and regenerated the expected activity of the enzyme towards BAEE.

The change in reactivity of the papain thiol group towards 2PDS at pH 3.8 consequent upon acid dena-

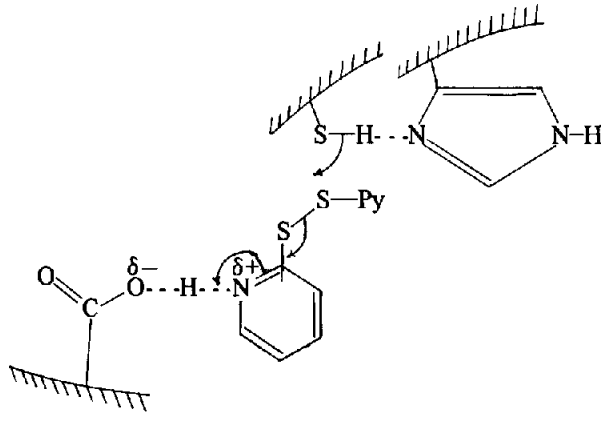
turation of papain at pH 2.6 and 25° was investigated. After ca. 1 hr the denaturation process, as measured by the fall in activity towards BAEE at pH 6.5, appeared to reach an equilibrium in which ca. 57% of the active papain originally present was inactive. Whilst reaction of papain with 2PDS at pH 3.8 at the beginning of the slow denaturation process was virtually instantaneous which is characteristic of the rate of reaction with active papain with 2PDS at the concentration employed ([2PDS] = 5×10^{-5} M), the reaction of the equilibrium mixture of active and inactive enzyme with 2PDS was clearly biphasic. A virtually instantaneous reaction was followed by a much slower reaction which reached an infinity reading which corresponded to the total thiol content of the inactive and active enzyme mixture. The initial portion of the slow phase of the reaction was extrapolated to zero time to give the stoichiometry of the initial fast phase. The correspondence between the fall in catalytic activity towards BAEE and the fall in the stoichiometry of the initial fast reaction with 2PDS demonstrates that the thiol group of papain shows a markedly higher reactivity towards 2PDS at pH 3.8 than does the thiol group in the acid-denatured enzyme.

In some of the papain preparations used, the fast reaction of the enzyme with 2 PDS at pH 3.8 was followed by a much slower reaction. The extent of the slow phase was ca. 5–10% of the total absorbance change and presumably represents reaction of small amounts of denatured enzyme or enzyme fragments.

The rate constants for the reaction of 2PDS with papain, L-cysteine and 2-mercaptoethanol at pH 3.8 given in the table suggested that it should be possible to estimate papain in the presence of these thiols. By extrapolating the initial portion of the slow second phase of the reaction (reaction of 2PDS with the low molecular weight thiol) to zero time it was found possible to titrate active papain in the presence of L-cysteine up to ca. 10 times molar excess of the latter and in the presence of 2-mercaptoethanol up to ca. 100 times molar excess.

It would appear that in its reaction with papain at low pH, 2PDS constitutes a fortuitous active site directed inhibitor which possesses considerable advantages over NBT as a titrant. Because 2PDS is an inhibitor and not a substrate it may be used to titrate papain more conveniently over a much wider range of enzyme concentrations. The titration may be carried out in the presence of other thiols including enzyme which although denatured, retains a reactive thiol group. The titration may be carried out conveniently at a higher pH (ca. 4) and 2PDS is sufficiently water soluble (ca. 1.5×10^{-3} M) to make unnecessary the inclusion of organic solvent in the assay solution.

The unexpectedly fast reaction of the papain thiol group with 2PDS at low pH is presumably the result of specific interactions of 2PDS with the enzyme in or near its active site. This suggestion is strengthened by the finding that the rate of reaction with 4,4′-dipyridyl-disulfide (4PDS) at low pH is not markedly different from the rates of reaction of this reagent with low molecular weight thiols. One interpretation which would rationalise the data is that the bell-shaped rate-pH profile at low pH for the reaction of the papain thiol group results from binding of one of the pyridine rings of 2PDS to the carboxyl group of aspartate 158 by hydrogen bonding. It is tentatively suggested that as a result of this binding a small conformational change occurs which results in the formation of a strong hydrogen bond between the thiol group of cysteine 25 and the imidazole group of histidine 159. This interaction would permit the reaction of the thiol group of cysteine 25 as a nucleophile at pH 4 by general base catalysis. One version of this mechanism in which the enzyme's thiol group is shown to attack one of the two possible electrophilic centres (S atoms) of the reagent, is presented schematically in (I).



Py = *o*-pyridyl

(I)

Addition of one more proton to the system, either to the carboxyl-pyridine pair or to the hydrogen bonded imidazole would result in the fall in rate observed below pH 3.75. Another possibility is that the general base catalysis of the reaction of the enzyme's thiol group is occasioned by the other pyridine ring of the reagent. If this is the case, it would appear that either general acid catalysis by the aspartate carboxyl group is required to operate simultaneously or the "solvent environment" provided by the enzyme's active site is required to give a fast reaction at low pH. One or both of these additional factors would need to be postulated as a requirement because the reactions of simple thiols with 2PDS, in which general base catalysis by one of the pyridine rings could presumably operate, are not abnormally fast at low pH.

A detailed kinetic and mechanistic study of the reactions of 2PDS, 4PDS and DTNB with papain, and other thiol proteases, is in progress.

Acknowledgements

We thank the M.R.C. for a research studentship to G.L. and for providing a Cary 15 Spectrophotometer and the S.R.C. for providing a Cary 16K Spectrophotometer.

References

[1] M.L.Bender, M.L.Begué-Cantón, R.L.Blakeley, L.J.Brubacher, J.Feder, C.R.Gunter, F.J.Kézdy, J.V.Killheffer, T.H.Marshall, C.G.Miller, R.W.Roeske and J.K.Stoops, J. Am. Chem. Soc. 88 (1966) 5890.
[2] B.J.Finkle and E.L.Smith, J. Biol. Chem. 230 (1958) 669.
[3] I.E.Liener, Biochim. Biophys. Acta 53 (1961) 332.
[4] M.R.Hollaway, A.P.Mathias and B.R.Rabin, ibid 92 (1964) 111.
[5] G.L.Ellman, Arch. Biochem. Biophys. 82 (1959) 70.
[6] C.W.Wharton, E.M.Crook and K.Brocklehurst, European J. Biochem. 6 (1968) 565.
[7] B.R.Baker, Design of Active-Site Directed Irreversible Inhibitors (John Wiley, New York, 1967).
[8] J.R.Kimmel and E.L.Smith, Biochemical Preparations, Vol. 6 (John Wiley, New York, 1958) p. 61.
[9] D.R.Grassetti and J.F.Murray, Arch. Biochem. Biophys. 119 (1957) 41.
[10] S.S.Husain and G.Lowe, Biochem. J. 116 (1970) 689.
[11] J.Drenth, J.N.Jansonius, R.Kockock, L.A.A.Sluyterman and B.G.Wolters, Phil. Trans. Roy. Soc. London Ser. B 257 (1970) 231.