The Reaction of Papain with Ellman's Reagent [5,5'-Dithiobis-(2-nitrobenzoate) Dianion]

By K. BROCKLEHURST, M. KIERSTAN and G. LITTLE*

Department of Biochemistry and Chemistry, St. Bartholomew's Hospital Medical College,
 University of London, Charterhouse Square, London EC1M 6BQ, U.K.

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1. The report by Robyt *et al.* (1971) that the 2-nitro-5-mercaptobenzoate dianion (Nbs_2^{2-}) produced by reaction of papain with the 5,5'-dithiobis-(2-nitrobenzoate) dianion (Nbs_2^{2-} ; Ellman's reagent) cleaves the three disulphide bonds in papain is shown to be incorrect. 2. When partially active papain containing approx. 0.4 mol of thiol/mol of protein is incubated with excess of Nbs_2^{2-} at pH 8, Nbs_2^{2-} reacts with the protein in an amount stoichiometric with the cysteinyl thiol group of papain to produce Nbs_2^{2-} in an amount stoichiometric with the original papain cysteinyl thiol group, and the catalytically inactive mixed disulphide, papain- Nbs_2^{2-} . 3. Papain catalyses the hydrolysis of Nbs_2^{2-} at pH 10.5 probably by nucleophilic catalysis involving the enzyme's thiol group. 4. These results cast very serious doubts on the claim by Robyt *et al.* (1971) to have established a new general method for the determination of cystinyl disulphide residues in proteins.

During the last 20 years the use of aromatic disulphides to detect and determine spectrophotometrically thiol groups in protein preparations has become widespread. The first study of this type was made by Barrett & Seligman (1952), who used 2,2'-dihydroxy-6,6'-dinaphthyl disulphide to demonstrate protein-bound thiol groups histochemically. Ellman (1958) reported the use of bis-(*p*-nitrophenyl) disulphide for thiol-group determination and later (Ellman, 1959) enhanced its water solubility by introduction of carboxyl groups into the benzene rings. The extensive use of Ellman's (carboxylated) reagent, 5,5'-dithiobis-(2-nitrobenzoic acid) (Nbs_2) in protein-modification studies has been reviewed (Glazer, 1970). The reaction of a cysteinyl thiol group in a protein with Nbs_2 is regarded generally as occurring by nucleophilic attack of the thiolate ion on the disulphide bond of the dianion, Nbs_2^{2-} , to produce, at pH values of about 8, a mixed disulphide and the dianion of 2-nitro-5-mercaptobenzoic acid (Nbs_2^{2-}) [see reaction (1), Scheme 1]. When a moderate excess of Nbs_2^{2-} is used at pH 8 or above, reaction (1) is effectively irreversible and the concentration of Nbs_2^{2-} at equilibrium, detected by its u.v. absorption at 412 nm, is a measure of the concentration of reactive cysteinyl residues in the protein.

It has been suggested (Robyt *et al.*, 1971), however, that reaction (1) may not describe adequately the reactions of proteins with Nbs_2^{2-} and that at least in some cases, e.g. papain, the reaction results in the scission of the cystinyl disulphide bonds of the protein.

Robyt *et al.* (1971) reported that, at pH 8, papain

* Present address: Department of Biology, Queen Elizabeth College, London, W.8., U.K.

reacts with Nbs_2^{2-} to provide Nbs_2^{2-} to the extent of 1.0 mol/mol of protein. They also reported that this production of Nbs_2^{2-} arises not merely from the reaction of papain's only thiol group, that of cysteine-25, which is part of the catalytic site (reaction 1), but also by the reactions (2)–(7) (Scheme 1), which involve reactions of Nbs_2^{2-} with the three cystinyl disulphide bonds (see Light *et al.*, 1964) of papain.

This would be surprising since Nbs_2^{2-} would be expected to be of only low nucleophilic reactivity. This may be inferred from (i) the low $\text{p}K_{\text{a}}$ of its conjugate acid (4.4; K. Brocklehurst & G. Little, unpublished work), which approximates to the $\text{p}K_{\text{a}}$ of its thiol group, (ii) the fact that the equilibrium in reaction (1) lies to the right ($K_{\text{equil.}}$ = approx. 14 at 25.0°C and $I = 0.1$; K. Brocklehurst and G. Little, unpublished work) and (iii) the value of $\lambda_{\text{max.}}$ of Nbs_2^{2-} (412 nm) compared with that of the corresponding monoanion in which the thiol group is protonated (approx. 325 nm) and with that of Nbs_2^{2-} (approx. 325 nm) (Donovan & White, 1971). These properties result from the resonance stabilization of Nbs_2^{2-} :

Robyt *et al.* (1971) used an activated, activator-free, solution of papain prepared from the 2 × crystallized product of Worthington Biochemical Corp. (Freehold, N.J., U.S.A.). As these authors point out, commercial preparations of papain are prepared by the method of Kimmel & Smith (1954). That Nbs_2^{2-} reacts with papain so prepared to provide 1.0 mol of Nbs_2^{2-} /mol of protein is remarkable. Commercial

Reaction as in (2) but with the second cystinyl disulphide bond (4)

Reaction as in (3) but with the thiol produced by cleavage of the second cystinyl disulphide bond (5)

Reaction as in (2) but with the third cystinyl disulphide bond (6)

Reaction as in (3) but with the thiol produced by cleavage

Ar represents $\text{O}_2\text{N}-\text{C}_6\text{H}_4-\text{CO}_2^-$ and P represents the part of the papain molecule not shown as functional groups.

Scheme 1. Reactions of (i) the cysteinyl thiol group and the three cystinyl disulphide groups of papain with Nbs_2^{2-} and (ii) $\text{papain-(Nbs}^-\text{)}_7$ with HO^- postulated by Robyt et al. (1971)

preparations of papain (including those of Worthington Biochemical Corp.) and those prepared in the laboratory from papaya latex by the method of Kimmel & Smith (1954) are generally found to contain 0.4–0.6 mol of thiol/mol of protein, as determined both by titration with Nbs_2^{2-} and by other methods (see, e.g., Sluyterman, 1967; Brocklehurst & Little, 1970; Hinkle & Kirsch, 1970). This applies to papain activated by incubation with and subsequent removal of either low-molecular-weight thiols (e.g. Brocklehurst & Little, 1970) or KCN (Klein & Kirsch, 1969). The latter method of activation was used by Robyt *et al.* (1971).

If the claim by Robyt *et al.* (1971) that the release of Nbs^{2-} from Nbs_2^{2-} , stoichiometric with the thiol content of papain, occurs by reactions (1)–(7) (Scheme 1) could be substantiated, it would have important consequences for studies in which papain is allowed to interact with either disulphides or thiols. This includes all studies on commercial papain preparations, since the enzyme is extracted from the latex in the presence of L-cysteine and is often activated by incubation with low-molecular-weight thiols. The potential occurrence of reactions of the type (1)–(7) is not restricted to papain. Robyt *et al.* (1971) claim to have demonstrated this series of reactions for *Aspergillus oryzae* α -amylase and also for denatured lysozyme after initiation of the reaction sequence analogous to (2)–(7) by generation of Nbs^{2-} *in situ*, and point out the generality of this reaction sequence for proteins containing cysteine and cystine residues. Because of the potential general importance of the suggested existence of reactions (2)–(7) and because the findings of Robyt *et al.* (1971) bear upon our studies on the kinetics of reactions of papain with aromatic disulphides (K. Brocklehurst & G. Little, unpublished work), we have re-examined the reaction of papain with Nbs_2^{2-} .

Robyt *et al.* (1971) infer the existence of reactions of the type (1)–(7) from two types of experiment, only one of which was carried out on the papain– Nbs_2^{2-} reaction. Papain was allowed to react with Nbs_2^{2-} at pH 8.1 for 30 min at room temperature (approx. 22°C). This resulted in the release of 1.0 mol of Nbs^{2-} /mol of protein as measured by the increase in u.v. absorption at 412 nm. The pH of the reaction mixture, consisting of buffer, papain– Nbs^- , Nbs_2^{2-} and Nbs^{2-} , was then changed from 8.1 to a higher value (usually pH 10.5) by addition of 0.1 M-NaOH. After 1–2 min the pH was readjusted to 8.1 by addition of 0.1 M-HCl and the E_{412} was recorded again. The increased amount of Nbs^{2-} detected after exposure of the protein (and the excess of Nbs_2^{2-}) to NaOH, after correction for alkaline hydrolysis of Nbs_2^{2-} (measured separately in the absence of protein) was interpreted in terms of reactions (2)–(7).

Unfortunately, this type of experiment is ambiguous. The increased quantities of Nbs^{2-} found after

the alkali treatment of the reaction mixture could be due to reactions other than those given in (2)–(7), e.g. protein-catalysed alkaline hydrolysis of Nbs_2^{2-} . A less ambiguous type of experiment is that which was carried out only for the *Aspergillus oryzae* α -amylase– Nbs_2^{2-} reaction but which it is tacitly assumed would apply also in the papain– Nbs_2^{2-} reaction. In this type of experiment the enzyme is allowed to react with Nbs_2^{2-} at pH 8 and the protein is then separated from the liberated Nbs^{2-} and excess of Nbs_2^{2-} on Sephadex. The amount of Nbs^{2-} bound to the protein is then determined by measurement of E_{412} after reduction with dithiothreitol. For the *Aspergillus oryzae* α -amylase– Nbs_2^{2-} reaction, Robyt *et al.* (1971) found that 1 mol of Nbs^{2-} /mol of protein was released by reaction at pH 8, and a further 9 mol of Nbs^{2-} /mol of protein were released by treatment with dithiothreitol after the Sephadex fractionation. These findings were interpreted in terms of reactions (1)–(7) (Scheme 1) and are in agreement with the known thiol (1 mol/mol) and disulphide (4 mol/mol) contents of *Aspergillus oryzae* α -amylase (Seon *et al.*, 1965). We cannot account at present for these results in the *Aspergillus oryzae* α -amylase– Nbs_2^{2-} reaction; one possibility is that several molecules of Nbs_2^{2-} might be adsorptively bound such that they resist separation from the protein on Sephadex. It is, if anything, even more difficult to account for the findings that Robyt *et al.* (1971) report for the denatured lysozyme– Nbs_2^{2-} reaction. It seems remarkable that the cystinyl disulphide bonds of lysozyme are not cleaved by 2.5 mM- HO^- ion at 90°C but appear to be cleaved at 22°C by approx. 10 μM - Nbs^{2-} , which is a much weaker nucleophile. The reactions of Nbs_2^{2-} with α -amylase and with lysozyme require further study. We here report, however, experiments that demonstrate conclusively that papain reacts with Nbs_2^{2-} to produce Nbs^{2-} according to reaction (1) and that reactions (2)–(7) do not occur in the papain– Nbs_2^{2-} system at pH 8.0. We report also experiments that strongly suggest that the release of >1 equivalent of Nbs^{2-} at pH values near 10.5 in this system is due to nucleophilic catalysis by the papain cysteinyl thiolate ion.

It is necessary to point out that our experiments on the papain– Nbs_2^{2-} reaction cast very serious doubts on the claim by Robyt *et al.* (1971) to have discovered a new analytical method for the determination of the cystinyl disulphide bond content of proteins.

Materials and Methods

Papain

The enzyme used in this study was the 2 $\times$ crystallized product of BDH Chemicals Ltd., Poole, Dorset, U.K. Papain was activated with dithiothreitol and the activator then removed by passage through a Sephadex G-25 column. Deoxygenated deionized water

containing 1 mM-EDTA was used throughout. The thiol content (0.40–0.46 mol of thiol/mol) of this activated preparation measured by titration with Nbs_2^{2-} in phosphate buffer ($\text{KH}_2\text{PO}_4 + \text{NaOH}$), pH 8.0, I 0.1, is in good agreement with the thiol contents of papain preparations obtained from Worthington Biochemical Corp. (2 $\times$ crystallized product) and from BDH Chemicals Ltd. papaya latex (3 $\times$ crystallized product) by the method of Kimmel & Smith (1954). These other papain preparations have been used from time to time for other studies in this laboratory and their thiol contents measured by various titration procedures (see Brocklehurst & Little, 1970). The molecular weight of papain was taken as 20700 (Smith *et al.*, 1955) and ϵ_{280} for papain as 5.1×10^4 (Glazer & Smith, 1961).

Mercuripapain

This was the 2 $\times$ crystallized product of Koch-Light Laboratories Ltd., Colnbrook, Bucks. U.K.

α -N-Benzoyl-L-arginine ethyl ester

This was obtained as the hydrochloride from BDH Chemicals Ltd. and an aqueous solution of this was adjusted to pH 6.0 immediately before use.

5,5'-Dithiobis-(2-nitrobenzoic acid) (Nbs_2)

This was the BDH Chemicals Ltd. product recrystallized from acetic acid. The Nbs_2^{2-} resulting from cleavage of Nbs_2^{2-} was monitored spectrophotometrically by using Cary 15 and 16K spectrophotometers, thermostatically controlled at 25.0°C, and $\epsilon_{412} = 1.36 \times 10^4$ (Ellman, 1959) was used to calculate its concentration.

Enzymic activity

The esterolytic activity of papain was determined by measuring the initial rate of the hydrolysis of α -N-benzoyl-L-arginine ethyl ester by using a Radiometer pH-stat and 0.1 M-NaOH as titrant.

Results and Discussion

Reaction of papain with Nbs_2^{2-} at pH 8.0

The question of whether this reaction occurs by reaction (1) only or by reactions (1)–(7) (Scheme 1) was readily resolved as follows. A typical experiment is described and the results of three such experiments are summarized in Scheme 2. Papain [0.24 mmol of protein containing 0.106 mmol of thiol, i.e. 0.44 mol of thiol/mol of protein as determined by titration

Scheme 2. Summary of the evidence which demonstrates that papain reacts with Nbs_2^{2-} at pH 8.0 by reaction (1) only and not also by reactions (2)–(7) of Scheme 1

The standard errors are based on three determinations in each case.

with 2,2'-bipyridyl disulphide at pH 8.0 (see Brocklehurst & Little, 1970)] was allowed to react with Nbs_2^{2-} (0.64 mmol, i.e. 6.0 mol/mol of free thiol group or 2.64 mol/mol of protein) at pH 8.0 and the progress of the reaction was recorded at 412 nm. At the end of the reaction Nbs_2^{2-} had been liberated in an amount stoichiometric with the thiol content of papain (0.44 mol/mol of protein, i.e. 1.0 mol/mol of free thiol). The protein, monitored by measurement of E_{280} , was then separated from Nbs_2^{2-} and excess of Nbs_2^{2-} on a 20 cm $\times$ 2.4 cm Sephadex G-25 column with deoxygenated, deionized water containing 0.1 M-KCl and 1 mM-EDTA as eluent. The protein had no measurable activity towards α -N-benzoyl-L-arginine ethyl ester, but when dithiothreitol (100 mol/mol of protein) was added to the inactive protein, it both regenerated the expected papain activity towards α -N-benzoyl-L-arginine ethyl ester and liberated Nbs_2^{2-} in an amount essentially stoichiometric with the original thiol content of the enzyme (0.42 mol/mol of protein). This experiment demonstrates that either the reaction of papain with Nbs_2^{2-} proceeds only by reaction (1), and not also by reactions (2)–(7), or that if further reaction occurs the product of such reaction does not liberate Nbs_2^{2-} on treatment with dithiothreitol. The latter interpretation was eliminated by analysis of the mixture of Nbs_2^{2-} and excess of Nbs_2^{2-} that had been separated from the protein on the Sephadex column. This mixture was allowed to react with dithiothreitol (300 mol/mol of original Nbs_2^{2-}) at pH 8.0. The reaction produced a further 1.04 mmol of Nbs_2^{2-} , which shows that 0.52 mmol of the 0.64 mmol of Nbs_2^{2-} originally present had not reacted with the protein. The amount that had reacted (0.12 mmol) is essentially the same as the amount of thiol originally present in the protein (0.106 mmol). This result demonstrates that papain had reacted with Nbs_2^{2-} only in an amount stoichiometric with its thiol content, i.e. reaction (1), and that reactions (2)–(7) had not occurred.

Results leading to the same interpretation as that described above were obtained when papain was allowed to react with a 50-fold molar excess of Nbs_2^{2-} at pH 8.0 rather than with a six-fold excess. In this case, however, it is more difficult to distinguish reaction (1) from reactions (1)–(7) by analysis of the unchanged Nbs_2^{2-} .

Reactions at pH 10.5

Robyt *et al.* (1971) reported that when the product of the reaction of papain with an excess of Nbs_2^{2-} at pH 8 is incubated at pH 10.5, Nbs_2^{2-} is released in an amount greater than that which would be predicted from (a) the rate of cleavage of Nbs_2^{2-} by HO^- and (b) the thiol content of the protein, assuming that the reaction at pH 8 had occurred only by reaction (1). Since the experiments reported above demonstrate

Fig. 1. Progress curves for the formation of Nbs_2^{2-} from Nbs_2^{2-} at 25.0°C and I 0.1

The continuous lines are taken from progress curves recorded continuously on a Varian G-2000 recorder attached to a Cary 16K spectrophotometer; zero time refers to the time of addition of Nbs_2^{2-} to the reaction cell. (a) At pH 10.5 (sodium bicarbonate buffer) in the presence of papain (80 μM -protein); $[\text{Nbs}_2^{2-}]_0 = 0.175 \text{ mM}$; extrapolation (broken line) of the linear portion of the progress curve to $t = 0$ shows that the initial fast phase of the reaction gives rise to 32 μM - Nbs_2^{2-} . (b) At pH 8.5 (tris-HCl buffer) in the presence of papain (80 μM -protein); $[\text{Nbs}_2^{2-}] = 0.51 \text{ mM}$; the reading extrapolated to infinite time (broken line) gives the concentration of papain containing a reactive thiol group as 32 μM (i.e. 0.4 mol of thiol/mol). (c) At pH 10.5 in the absence of papain; $[\text{Nbs}_2^{2-}] = 0.175 \text{ mM}$.

that at pH 8 Nbs_2^{2-} is formed only by reaction (1), we carried out experiments to attempt to discover the reason for the enhanced rate of formation of Nbs_2^{2-} in the presence of papain- Nbs^- at pH 10.5.

That this is not due to 'non-specific' protein-catalysed cleavage of Nbs_2^{2-} was demonstrated by the fact that the rate of formation of Nbs_2^{2-} from Nbs_2^{2-} in sodium bicarbonate buffer, pH 10.5, I 0.1, was not increased by inclusion of approx. 0.1 mM-mercuripapain in the reaction mixture. We next considered the possibility that papain- Nbs^- might undergo alkaline hydrolysis at pH 10.5 more rapidly than Nbs_2^{2-} and in such a way as to provide papain thiolate ion, which could catalyse the formation of Nbs_2^{2-} from Nbs_2^{2-} by nucleophilic catalysis. That papain does catalyse the cleavage of Nbs_2^{2-} at pH 10.5 is demonstrated in Fig. 1. With enzyme present, the initial rapid release of Nbs_2^{2-} , stoichiometric with the thiol content of the enzyme, is followed by further release of Nbs_2^{2-} , which is faster than the alkaline hydrolysis of Nbs_2^{2-} in the absence of active papain.

Scheme 3. *Minimal reaction scheme for the catalysis of the alkaline hydrolysis of Nbs_2^{2-} by the papain thiolate ion*

At pH 10.5 a solution of the products of alkaline cleavage of Nbs_2^{2-} (approx. 0.1 mM) is yellow. This results from the absorption band of Nbs_2^{2-} , $\lambda_{\text{max.}} = 412 \text{ nm}$. At higher pH values (approx. 13–14), however, the system is more complex. The solution under these conditions is orange: a shoulder appears in the spectrum at approx. 500 nm. Ar and P are as identified in Scheme 1.

A minimal reaction scheme which could account for the papain-catalysed hydrolysis of Nbs_2^{2-} is given in Scheme 3. This is in accord with the known products of alkaline cleavage of Nbs_2^{2-} and the probable products of alkaline cleavage of aryl-alkyl mixed disulphides (see Donovan & White, 1971). If papain- Nbs^- is cleaved by HO^- to provide, at least in part, Nbs_2^{2-} , the catalytic effectiveness of papain would be expected to diminish as the reaction proceeds because of the progressive conversion of the enzyme into the inert sulphinic acid form, PSO_2^- . Mechanisms such as this therefore could account for the stoichiometric yield of Nbs_2^{2-} in the cleavage of papain- Nbs^- by HO^- in the presence of excess of Nbs_2^{2-} at pH 10.5 found by Robyt *et al.* (1971).

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