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A pulse radiolysis approach to fast reductive cleavage of a disulfide bond to uncage enzyme activity

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Running Title: Activation of Caged Enzyme by Pulse Radiolysis

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Abstract

The essential thiol of the enzyme papain has been caged by linking to an aromatic thiol. The resulting caged protein is inactive but enzymatic activity is fully restored upon chemical cleavage of the protective disulfide bond. We have exploited the chemistry of this disulfide bond to uncage papain by pulse radiolysis. We have shown that up to 10% of the enzyme activity can be restored by reductive pulse radiolysis. This approach has been tested on a small molecule model system, and experiments on this model compound show that pulse radiolysis of the mixed cystein-aromatic disulfide results in selective reduction of the disulfide bond to generate a thiol in 10-20% yield, consistent with the radiolytically restored activity of the caged papain quantified by the biochemical assay.

Keywords: Biological activity; Free radicals; Reductive pulse radiolysis; Papain; Uncaging

Introduction

The photorelease of active biological molecules from inactive, caged precursors is extensively employed for the study of dynamic processes in many biological systems. In the case of proteins, the methodology uses a photoactive compound to label a single amino acid site or various sites randomly [1,2]. Although photocaged proteins have been successfully used to study a variety of physiological processes, their practical applications are still limited [3-4]. One of the limitations lies in the choice of the photoremovable group. A recent study [5] estimates that 80% of the caged groups published are based on the nitrobenzyl chromophore. These compounds have limitations such as slow photorelease and formation of reactive nitroso compound byproducts [2,6].

We have developed a strategy for proteins with a new caging group that can be rapidly removed and does not release unwanted byproducts.

Our approach uses a disulfide bond to reversibly protect an active site thiol, and this paper explores the application of this approach to re-initiate the enzymatic activity of the

caged papain using reductive pulse radiolysis on the microsecond time-scale. Papain (EC 3.4.22.2), isolated from the latex of *Carica papaya*, is a cysteine protease with a single exposed cysteine residue (25) that is essential for enzymatic activity [7]. Papain is considered a model system for the study of sulphydryl enzymes and mammalian cysteine proteases that participate in many essential biological processes [8-11]. The thiol of cysteine 25 can react with low molecular weight disulfides via thiol/disulfide exchange [12,13]. The resulting papain disulfide is caged and shows no catalytic activity. Activity can be restored by cleaving the disulfide with, for example, a reducing agent such as dithiothreitol (DTT) to regenerate the essential thiol.

Laser photolysis is the method of choice to uncage proteins. However attempts to activate a disulfide-caged papain by photolysis were not successful. A possible explanation is that enzyme activity requires the presence of a thiol rather than the radical species that are produced by photolysis. We have attempted to regenerate thiols from thiyl radicals by flash photolysis of model disulfides in the presence of electron donors such as copper (I) chloride and vitamin E. However, thiols were not generated by this approach probably because the recombination of the radical pairs is too fast to

allow for the reduction to take place [14,15]. Pulse radiolysis is an alternative approach to induce heterolytic cleavage of the protecting disulfide bond and regenerate the active thiol of caged papain as shown in Figure 1 [16-25].

In reductive pulse radiolysis, carbon dioxide radical anions ($CO_2^{\bullet-}$) generated by the process illustrated in Fig. 1A react with disulfides at neutral pH to produce disulfide radical anions with rate constants of the order of 10^8 $M^{-1}s^{-1}$ [19-21,23,27,28]. In aqueous solution, the thiolate anion is in equilibrium with the corresponding thiol [19-21,22,23,26-28].

If the starting disulfide is not symmetric, two types of radical and thiol species can be formed from the reaction of $CO_2^{\bullet-}$ with the target disulfide. Reductive pulse radiolysis of a caged papain would lead to a cysteine thiyl radical or to the active thiol depending on which of the two pathways in Fig. 1B prevails. Reactions of $CO_2^{\bullet-}$ at protein sites different from the target disulfide cannot be excluded and the relative efficiency of all these reactions will determine the yield of protein thiyl radicals and the yield of the active species, the protein thiol [24,25,29].

Approach

The mixed disulfide of papain, and *p*-aminothiophenol (Fig. 1B and Fig. 2A) was chosen for the reductive pulse radiolysis

study. The *p*-aminophenyl thiyl radical has a strong characteristic absorption at 600 nm, where there is little contribution from the protein or any protein products generated by pulse radiolysis [31-35]. The extinction coefficient of this radical [36,37] is nearly two orders of magnitude larger than that of the alkyl thiyl radicals in polar solvents (ε_{360nm}~300 $M^{-1}cm^{-1}$) [38]. Thus the formation of the aromatic radical should be easy to monitor spectroscopically, which provides an indirect probe to estimate the yield of cysteine thiol from pulse radiolysis. If pathway 2 in Fig. 1B is followed, one cysteine thiol is generated for every aromatic radical. Formation of the cysteine radical and *p*-aminothiophenol via pathway 1 is spectroscopically silent in the 500-600 nm region and will not interfere with the measurements [36-38]. In order to find suitable conditions for the caging and activation of papain by pulse radiolysis, we prepared model compound (**3**) (Fig. 2B). In the model compound, the $CO_2^{\bullet-}$ reacts only with the disulfide bond, and this system can be used to estimate the yield of thiol in the absence of the alternative pathways that are likely to occur in the presence of papain.

Experimental Procedures

Materials and Instruments

Chemicals and solvents were obtained from commercial sources (Sigma, Aldrich, Pharmacia and Merck). They were of the highest purity available and used without further purification unless otherwise noted. Twice crystallised papain (EC 3.4.22.2,) from the latex of *Carica papaya* was purchased from Sigma. Vivaspin centrifugal devices (Mw cut off = 10000, polyethersulphone membrane, 20 mL capacity) were purchased from Sartorius/Vivascience. All solutions were filtered on a 0.2 μm filter (Amicon). TLC was carried out using Merck Silica Gel 60 (F_{254}) pre-coated aluminium sheets. Flash chromatography was performed using BDH Silica gel 60 (40-63 μm).

NMR spectra were recorded on Bruker AMX-250, AMX-400. The following abbreviations are used for the multiplicities: s = singlet, d = doublet, t = triplet, q= quartet, m= multiplet, br = broad.

The pulse radiolysis experiments were carried out with a 12 MeV RDL, 3GHz electron linear accelerator at Free Radical Research Facility, Daresbury,UK [39]. Both the normal mode and the single pulse mode were used with a pulse duration from 0.22 to 2μs and with a peak current of about 30 mA The sample cell for flow system experiments, constructed from Spectrosil quartz, had an optical path length of 25 mm [40]. Absorbed

doses were determined from the transient $(SCN)_2^{\bullet-}$ formation in air-saturated 10^{-2} M KSCN as described by Adams *et al.* [41] but using the updated $G\varepsilon$ value of 2.59 x 10^{-4} m^2 J^{-1} obtained by Buxton and Stuart, *G* being the radiation chemical yield of $(SCN)_2^{\bullet-}$ and ε its molar absorption coefficient at 475nm [42]. Saturation of such solutions with N_2O results in a doubling of the $(SCN)_2^{\bullet-}$ yield.

Activation of papain

Twice crystallized papain (1mg/mL) was incubated in 200 mM formate buffer pH 6.2, 20 mM DTT for 3 h. The solution was loaded on a Sephadex G-25 column and eluted in the same buffer with no DTT. The fraction containing protein was dialysed in deoxygenate formate buffer and subsequently used for enzymatic activity assay or for reaction with (**3**). Protein concentration was calculated using ε_{280} = 56185 M^1s^{-1} [43].

Determination of free thiol group and papain activity

Protein activity was determined spectrophometrically at 410 nm using the chromogenic substrate N-benzoyl-L arginine-*p*-nitroanilide hydrochloride (L-Bapna) [44]. In a typical assay 8 μM protein, 400 μM L-Bapna in 200 mM formate pH 6.2 were used. The enzyme activity was unaffected by addition of DTT (2mM) or

acetonitrile (2% v/v). Measurements of enzyme activity in 50 mM phosphate buffer, 1 mM of ethylenediaminetetracetic acid (EDTA), 100 mM NaCl were carried out in the same experimental conditions. The free thiol content of the protein (8 μM) was determined spectrophotometrically at 412 nm using the chromogenic substrate 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB)(ε_{410} = 12750 $M^{-1}cm^{-1}$) in 200 mM formate pH 6.2 [45].

Synthetic Procedures

*Synthesis of (**2**)*.To a solution of 0.7 g (3.17 mmol) of 2-2′ dipyridyl disulfide in 21 mL of a mixture of ethanol/acetic acid 20:1 v/v, were added 0.44 g (3.17 mmol) of 4-aminothiophenol and the solution was stirred for 1 h. The solvent was then removed under reduced pressure and the remaining solid redissolved in CH_2Cl_2 (30 mL). The solution was washed with a saturated solution of $NaHCO_3$ in water (5 x 20 mL), dried with anhydrous Na_2SO_4 and the solvent removed under reduced pressure. The residue was purified by flash chromatography on silica, using a mixture chloroform/acetic acid/THF 95:3:2 v/v. The fraction containing (**3**) was washed with a saturated solution of $NaHCO_3$, dried with Na_2SO_4, and the

solvents were removed under reduced pressure (0.31 g, 41.7% yield).

^{1}H-NMR (250 MHz, $CDCl_3$): $\delta = 8.47$ (d, J = 3.97, 1H), 7.76 (d, J = 7.95, 2H), 7.63 (m, 1H), 7.37 (d, J = 8.85, 2H), 7.07 (m, 1H), 6.58 (d, J = 8.55, 2H).^{13} C-NMR (75 MHz, $CDCl_3$): 160.5, 149.5, 147.2, 137.1, 132.5, 123.9, 120.6, 119.9, 115.5. MS (CI^+, gas NH_3) m/z: 112, 124, 235(MH^+). $C_{11}H_{10}N_2S_2$ requires 234.34.

*Synthesis of (**3**).* The same procedure described for syntheses of (**2**) was used, starting from 0.15 g (0.6 mmol) of (**2**) and 0.1 g (0.6 mmol) of N-acetyl-L-cysteine. The residue was purified by crystallisation from chloroform/ethanol (0.13 g, yield: 75%).

^{1}H-NMR (250 MHz, CD_3OD): 7.35 (d, J = 8.14, 2H), 6.7 (d, 2H, J = 7.94), 4.79 (dd, 1H), 3.24 (dd, 1H), 2.99 (dd, 1H), 2.00 (s, 3H). ^{13} C-NMR (DMSO*d*): 177.2, 171.3, 146.0, 133.0, 125.1, 116.0, 53.4, 43.5. HRMS (ES^+) m/z: = 287.1066 (MH^+), $C_{11}H_{14}N_2O_3S_2$ requires 287.1047. UV/Vis. (50 mM HCOOH/HCCOONa, pH 6.2): ε_{251}= 11080 $cm^{-1}M^{-1}$, ε_{280}= 6926 $cm^{-1}M^{-1}$.

Synthesis of modified papain. A solution of active (DTT free) papain (30 μM) in 200 mM of deoxygenated 200 mM formate pH 6.2 was added under stirring to a solution of (**3**)(0.047 M) in acetonitrile to give 800 μM of (**3**) in the reaction mixture. The reaction was left under N_2 and stirring for 3 h. After 2 h the solution become turbid and the supernatant did not show any enzymatic activity upon addition of L-Bapna. After 3 h the reaction mixture was centrifuged and the supernatant dialysed to remove excess of (**3**). The concentration of modified protein was estimated by using ε_{280} = 58011 $M^{-1}cm^{-1}$ (ε_{280} modified papain= ε_{280} of native papain + ε_{280} of (**3**) - 1/2 ε_{280} of 2-2′ dipyridyl disulfide) [43]. The yield of the reaction was 37% and the maximum achievable concentration of modified papain in solution was 20 μM. This was tested by centrifugal dialysis.

Pulse Radiolysis

Samples for time resolved experiments were prepared as follows: bis(*p*-aminophenyl) disulfide (500 μM) was dissolved in a 1:4 solution of isopropyl alcohol (IPA)/H_20 and the solution bubbled with N_2 for 30 min. Compound (**3**) (140 μM) was dissolved in 50 mM formate pH 6.2 and bubbled with N_2O for 30 min. Solutions of native papain (12 μM) and modified papain (12 μM)

in 200 mM formate pH 6.2, were saturated with N_2O by using a needle penetrating down to a few millimetres over the liquid surface avoiding bubbling and contact with the solutions [27]. Gas equilibration was carried out as follow: the solutions were stirred and subjected to partial vacuum that was broken with N_2O for at least 4 times in the course of 30 min. For the flow experiments, the reservoir where the solutions were deareated was then connected to the irradiation cell under a stream of N_2O. The flow rate was adjusted to ensure the irradiation of a fresh portion of solution by each pulse.

The preparation of a typical sample for continuous radiolysis (uncaging) was carried out as follows: L-Bapna or DTNB (1.2 mL) and protein or disulfide (1.2 ml) in formate pH 6.2 were added to two cuvettes (quartz, 1 cm path length) equipped with tubes. The two tubes were joined and equipped with a Young tap and a connector. In this way the solutions were maintained separated but were simultaneously subjected to partial vacuum and subsequent N_20 equilibration. Only the cuvette containing protein or disulfide was subjected to continuous radiolysis. In control experiments formate pH 6.2 and L-Bapna solutions were radiolysed and prepared as described for the protein samples. Control experiments showed that the pH of the formate solution was not alterated by irradiation.

Doses of 50-800 Gy were delivered by exposing the solutions at dose rates of 20 Gy^{-1}. After approximately 5 min from radiolysis the protein or disulfide solutions were mixed with the L-Bapna or DTNB solutions respectively. Immediately after mixing, the activity of the protein and the thiol content of the disulfides were determined spectrophotometrically as described. For the protein samples, the activity was monitored up to 8 min after mixing and then after the addition of DTT (20 mM).

Results and Discussion

Synthesis

Model compound (**3**) was prepared as shown in Fig. 3. A thiol-disulfide exchange reaction between *p*-aminothiophenol and bis(2-pyridyl) disulfide (**1**) gave (**2**). The yield was low due to the reaction of (**2**) with unreacted (**1**), that led to the formation of bis(p-aminophenyl) disulfide, which was separated from (**2**) by column chromatography. Reaction of (**2**) with N-acetyl L-cysteine yielded the mixed disulfide(**3**). Caged papain was prepared in a similar manner using compound (**2**). A solution of sodium formate and formic acid, pH 6.2, was used in this

procedure in place of the standard solutions containing sodium chloride that are typically used to test papain activity [46]. Chloride anions were avoided because they compete with formate in the reaction with the hydroxyl radicals generated by pulse radiolysis [47,48]. Although the product of this reaction is unstable at pH 6.2 and chloride ions are re-generated, this is a competitive pathway that can reduce the yield of $CO_2^{\bullet-}$ [47,48]. The concentration of formate was chosen to give an ionic strength optimal for catalytic activity (conductivity, 1500 μScm^{-1})[46].

The efficiency of the caging reaction was evaluated using a standard assay for protease activity. Papain hydrolyses N-benzoyl-L arginine-*p*-nitroanilide hydrochloride (L-Bapna) to give *p*-nitroaniline [44]. As *p*-nitroaniline has a characteristic absorption band in the visible region at 410 nm, activity is quantified by the increase in optical density at 410 nm. In 200 mM formate, the activity of papain was similar to that observed in the standard phosphate/sodium chloride buffer. In the presence of DTT, which prevents oxidation of the catalytic thiol, there was no decay in papain activity over 24 hours in both the formate and phosphate solutions. Acetonitrile (2%) is required to dissolve a sufficient amount of compound

(**2**) in water to ensure a 10 fold excess with respect to protein in the caging reaction, but the presence of the organic solvent was found to have no effect on the catalytic activity of papain [43].

The protein mixed disulfide product showed no detectable catalytic activity, but on addition of DTT, the activity of papain was completely restored (Table 1). Thus the functionalisation of Cys25 is quantitative, and the active site is not irreversibly modified by the reaction.

Pulse Radiolysis of The Model Compound

Pulse radiolysis of (**3**) was carried out in N_20 saturated formate/formic acid (pH 6.2) which yields formate radicals as the reducing species upon irradiation (Fig. 1). The chemical processes expected in the course of radiolysis of (**3**) are shown in Fig. 4, and the UV/Visible absorption of the transient species formed, monitored in the 350-660 nm region, are shown in Figure 4A (-•-). Two new absorption bands were observed at 380 nm and at 600 nm. The time course for the decay of these bands is practically identical, suggesting that they belong to the same species (Fig. 5A inset). These spectra are very similar to those observed in flash photolysis experiments on bis(*p*-aminophenyl) disulfide, which suggests that pulse

radiolysis of (**3**) leads to the formation of the same *p*-aminophenyl thiyl radical [31,33,35-37]. Identical transient absorption spectra were obtained upon radiolysis of (**3**) under a variety of reducing conditions: in N_2-saturated methanol and N_2-saturated 10 mM sodium phosphate buffer (pH 7.0) with 1% *tert*-butanol.

To confirm the identity of the transient species responsible for the spectra shown in Figure 5A (-●-), we carried out pulse radiolysis experiments on bis (*p*-aminophenyl) disulfide. Due to the poor solubility of bis (*p*-aminophenyl) disulfide in water, experiments were performed in a 4:1 mixture of water and isopropyl alcohol (IPA). Under these conditions, IPA radicals and hydrated electrons are formed, but they are also reducing species that will behave like the $CO_2^{\bullet-}$ radicals. The chemical processes expected in the course of radiolysis of bis (*p*-aminophenyl) disulfide are shown in Figure 4B, and the spectra obtained are shown in Fig. 5A (-■-). The transient absorption spectra observed in the radiolysis of bis(*p*-aminophenyl) disulfide are very similar to those obtained in the pulse radiolysis of (**3**) (Fig. 5A, -●-) and in the flash photolysis of bis(*p*-aminophenyl) disulfide in polar solvents indicating that the *p*-aminophenyl thiyl radical is produced in all cases [31,32,36,37].

The two different dissociation pathways proposed in Fig. 1B are based on the formation of a disulfide radical anion as a primary product of reductive radiolysis. Aliphatic disulfide radical anions are reported to absorb in the range 420 – 450 nm, with lifetimes in water in the microsecond range for small, linear disulfides [26]. No such data are available for aromatic systems at room temperature. However, these species (attributed absorption maximum 427 nm) were formed by γ-radiolysis of phenyl and benzyl disulfides in a glass matrix of 2-Me-THF at 77 K [49]. This suggests that the lifetime of the aromatic disulfide radical anions in fluid solution is considerably shorter than that of their aliphatic counterparts. The lack of absorption bands attributable to disulfide radical anion in the radiolysis of (**3**) and caged papain may be due to its lifetime being outside of the detection limit of our experiments. Alternatively, it is possible that the limiting step is a formation of disulfide radical anion, a fast dissociation of which would leave too low a stationary concentration to be detected.

The rate constants for the dissociation and recombination processes of transient species proposed in Figure 4 were determined by fitting the experimental data to the relevant kinetic models using Specfit [50] with the assumption that

there are no other significant processes that occur in this system. The transient absorption data for bis(*p*-aminophenyl) disulfide at 600 nm were fit to a simple three state model: formation of thiyl radical followed by geminate recombination of the radical to give the disulfide (Fig. 4B). Using the known concentrations of reducing species generated by radiolysis and the extinction coefficient of the *p*-aminophenyl thiyl radical [36,37], the rate constants shown in Table 2 were obtained. In the case of (**3**), the experimental data were fit to a more complex kinetic model that considered the two possible dissociation pathways and the three possible radical recombination processes (Fig. 4A). The number of variables required to fit the data was reduced by fixing the rate constant of recombination of two aromatic radicals at the value measured in the experiments on *p*-aminophenyl disulfide (2.4 x 10^9 $M^{-1}s^{-1}$) and the literature value for the rate constant for the recombination of two cysteine thiyl radicals (3.4x10^9 $M^{-1}s^{-1}$) [38]. The values determined for the remaining rate constants are shown in Table 2, and the quality of the fit is illustrated in Fig. 5A(inset).

The kinetic parameters in Table 2 allow us to calculate speciation profiles for the products of the pulse radiolysis experiments (Figs. 5B and 5C). Extrapolation of the decay of

the aromatic thiyl radical absorption at 600 nm to time zero allows us to estimate the total yield of aromatic thiyl radical based on the concentration of the reducing species which is the limiting starting material (yield of thiyl radical = 17 %). The populations in the speciation profiles are all shown relative to the concentration of reducing species generated by radiolysis. Pathway 2 in Figure 4A is clearly the dominant process for dissociation of the proposed mixed disulfide radical anion. Due to radical recombination following the dissociation process, a maximum is observed in the concentration of aromatic thiyl radicals after 50 μs. The thiol/thiolate products are not subject to a similar recombination process, and if the reactions illustrated in Figure 4A are the major processes in this system, one can estimate the final yield of alkyl thiol as 17% (Fig. 5C).

A possible explanation for the selectivity observed in the dissociation of the disulfide radical anion may be due to the higher pKa value of N-Acetyl L-cysteine compared to *p*-aminothiophenol (pKa 9.52 *vs* 8.75 respectively), indicating that the aromatic thiolate anion is a better leaving group due to its ability to stabilise negative charge [45,51].

The kinetic analysis on (**3**) suggests that it should be possible to recover up to 17% of the total enzyme activity of caged papain using pulse radiolysis, provided that all of the disulfide can be reduced with a sufficient dose of radiation. Repeated irradiation was therefore tested using model compound (**3**) to quantify the maximum yield of thiol that can be generated. After radiolysis of a 20 μM sample of (**3**) with a dose equivalent to 240 μM of $CO_2^{\bullet-}$ (400 Gy), the solution was analysed for the presence of thiols using Ellman's reagent [52]. The results showed that the amount of active thiol generated was 20 μM, i.e. a quantitative conversion of disulfide to thiol. This experiment suggests that secondary reactions involving thiol/thiolate moieties are not important in the radiolysis of (**3**).

Pulse Radiolysis of Caged Papain

The radiolytic dose was adjusted according to the maximum solubility of caged papain (12 μM) to achieve a stoichiometric ratio of protein to $CO_2^{\bullet-}$ radical anions. The transient UV/Visible absorption spectra recorded in the course of the radiolysis (Fig. 6) show two new absorption bands at the same position as for model compound (**3**), 380 nm and 600 nm. There is a difference in the relative intensity of the two absorption

bands compared with the results obtained for (**3**): the absorption at 380 nm is higher in the case of the protein. In addition, this band is broad and extends up to 500 nm, where no absorption was observed for (**3**). This suggests that there are additional processes that occur in the protein that are responsible for the absorption between 400 and 500 nm [27,33-35,53].

The decay of the absorption at 600 nm can be used to quantify the amount of aromatic thiyl radicals generated in this experiment, because the rate of its bimolecular recombination has been measured in the control experiments described above. Extrapolating the decay at 600 nm to time zero gives the yield of 8% of aromatic thiyl radicals based on the concentration of papain (or reducing species that are present in equal amounts). This value provides an estimate of the active papain thiol formed in the pulse radiolysis of the caged protein, which is lower than the 17% found for pulse radiolysis of the model small molecule (**3**). This reflects the presence of alternative pathways for the reaction of the protein with $CO_2^{\bullet-}$, which compete with the reduction of the disulfide bond and may lead to the extra features present in the transient absorption spectra of the radiolysed protein between 400 and 500 nm [27,33-35,53].

A similar band is observed upon irradiation of proteins containing disulfides and is attributed to the corresponding disulfide radical anions [18-20,23,33]. The difference in behaviour of caged papain relative to these proteins, is probably due to the more rapid dissociation of the radical anion of the non-native disulfide relative to the radical anions of native disulfides which are conformationally trapped by the folded proteins. This is supported by the observation that no band at 400-500 nm was found for radiolysis of (**3**) or for *p*-aminophenyl disulfide. Assignment of the absorption in the 400-500 nm range to the disulfide radical anion of the caging disulfide is unlikely, as kinetic analysis shows that the 600 nm absorption due to the aromatic thiyl radical is not formed from the absorption band in the 400-500 nm range. We therefore conclude that the absorption band at 400-500 nm arises in our experiments from the radical anions of the three native disulfide bonds of the caged papain as previously observed for radiolysis of papain under similar experimental conditions [33,35]. This process is one of the alternative pathways that accounts for a lowering of the yield of uncaged, active papain relative to the model disulfides [27,33-35,53]. Nevertheless the 8% yield of thiyl radicals from caged papain determined by taking into account only the decay of the

absorption at 600 nm, is virtually identical to the radiolytically induced activity (see below).

To improve the yield of uncaged active papain, the caged protein (12 μM) was subjected to radiolytic doses corresponding to a 2-40 fold excess of $CO_2^{\bullet-}$ radicals. The products of radiolysis were analysed using the L-Bapna assay. Excess L-Bapna was used to ensure pseudo-first order conditions, and the increase in absorbance at 410 nm was linear with time, giving a direct measure of enzyme activity. To measure the amount of unreacted caged papain remaining after radiolysis, DTT was added to the products, and the increase in enzyme activity was measured. The results are shown in Table 1.

For radiolytic doses of 50-130 Gy corresponding to a 5-12 fold excess of $CO_2^{\bullet-}$ radicals, there was no detectable enzyme activity. When DTT was added to these solutions, 80-90% of the enzyme activity was recovered. At a radiolytic dose corresponding to a 20 fold excess of $CO_2^{\bullet-}$ radicals (400 Gy), 10% enzyme activity was induced by radiolysis, but this increased to only 40% on addition of DTT. This observation suggests that a significant fraction of the protein has been irreversibly damaged by radical reactions at this dose. For

radiolytic doses of 600-800 Gy, corresponding to a 30-40 fold excess of $CO_2^{\bullet-}$ radicals, a precipitate was observed in the solution immediately after exposure, indicating degradation of the protein.

A control experiment was carried out using native papain subjected to a dose of 400 Gy. There was no detectable enzyme activity after radiolysis, and only 8% activity was recovered on addition of DTT compared to the 40% activity recovered from the caged protein. This result indicates that the presence of the aromatic disulfide at the active site of the caged protein affords protection from radiolytic damage.

Conclusions

We have used a selective disulfide bond formation to reversibly cage the cysteine protease papain. We have provided evidence that reductive pulse radiolysis can be used to uncage the enzyme on the microsecond timescale to recover up to 10% of the activity with further 30% being regenerated by standard chemical means.

The use of p-aminophenyl disulfide as caging group allows observation of the formation of the aromatic thiyl radical on reductive radiolysis of caged papain in formate solutions. There is a clear correlation between the yield of aromatic

thiyl radical and the recovered activity of the enzyme. However the yield of thiyl radicals from caged papain is less than expected from analogous experiments on model disulfides. This indicates that some radiation damage occurs in caged papain lowering the yield of uncaging. However, radiolysis of native papain reduced the activity to 8% of the starting value. This is substantially lower than the 40% recovery in enzyme activity observed in the case of caged papain. This observation suggests that the caging disulfide bond protects the enzyme from radiation damage. The extent of the radiation damage is likely to be different depending on the protein, because the relative surface exposure of the aminoacid residues and their role in determining the enzyme activity will differ. Nevertheless the approach is novel and the 10% recovery of activity by radiolysis is encouraging. An attractive feature of this approach is that it can be applied to uncage sulphydryl enzymes on fast timescales with the prospect of time-resolved experiments on catalytic processes in the active site.

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ABBREVIATIONS

DTNB- 5,5′-dithiobis-(2-nitrobenzoic acid)

DTT- dithiothreitol

EDTA- ethylenediaminetetracetic acid,

IPA- isopropyl alcohol

L-Bapna- N-benzoyl-L arginine-*p*-nitroanilide hydrochloride

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Table 1. Observed pseudo first order rate constants for hydrolysis of L-Bapna. The activity is expressed as: (k_{obs} /k_{papain}) × 100.

Enzyme	Dose (Gray)	Before DTT		After DTT	
		k_{obs} (s^{-1}) [a]	Activity	k_{obs} (s^{-1}) [a]	Activity
papainSH	0	n.d. [b]	n.d.	76	100
papainSSaniline	0	0	0	71	93
papainSH	400	n.d.	n.d.	6	8
papainSSaniline	400	8	10	31	40
papainSSaniline	130	0	0	62	82

[a] The error in the measurements of k_{obs} is estimated to be 5%. [b] n.d., not determined.

Table 2. Rate constants for the processes following pulse radiolysis of bis(*p*-aminophenyl) disulfide (Fig.6) and (**3**) (Fig. 8). The error in the measurements of k_{obs} is estimated to be 10%.

Disulfide	k_1 ($M^{-1}s^{-1}$)	k_3 ($M^{-1}s^{-1}$)	k_2 ($M^{-1}s^{-1}$)	k_{rec1} ($M^{-1}s^{-1}$)	k_{rec2} ($M^{-1}s^{-1}$)	$k_{rec'}$ ($M^{-1}s^{-1}$)
***p*-aminophenyl**	-	2.6×10^4	-	2.4×10^9	-	-
(**3**)	4.6×10^3	-	2.2×10^4	2.4×10^9	3.4×10^9[a]	3.1×10^9

[a] Taken from reference [49].

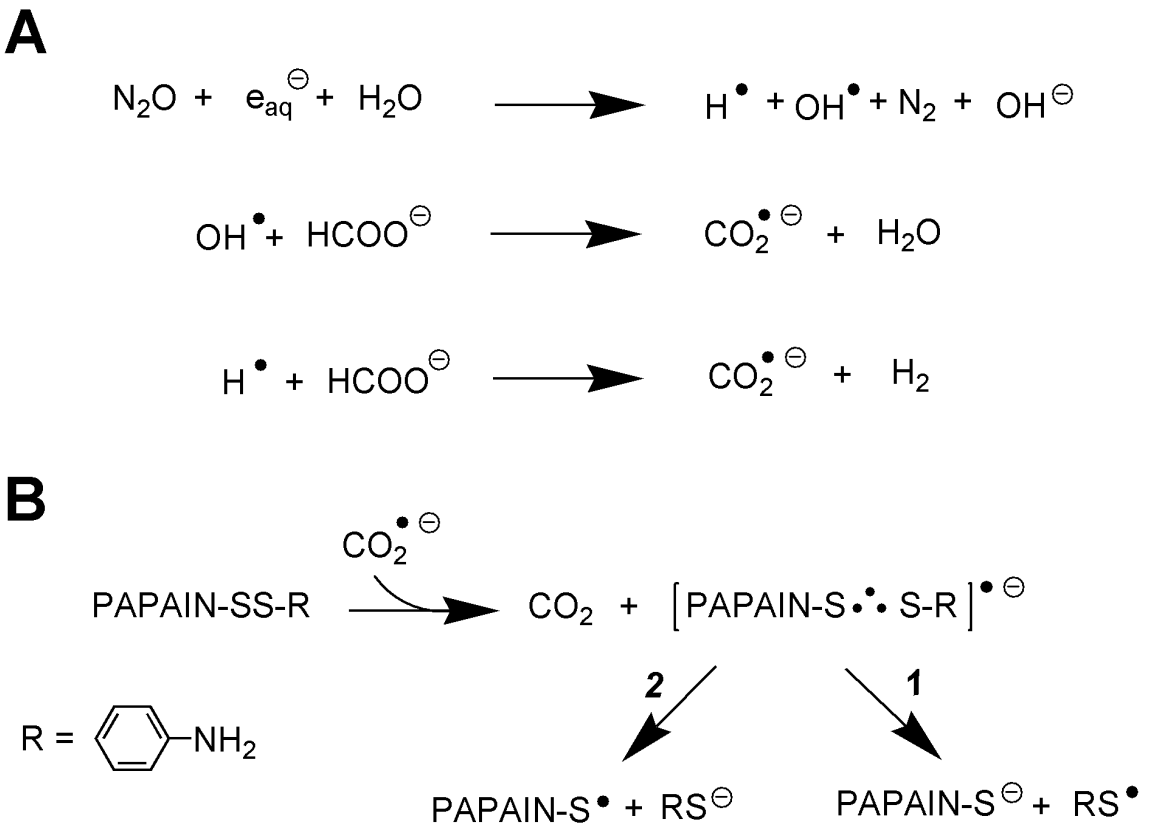

Figure 1. A) Reactive species formed in the pulse radiolysis of a solution of formate saturated with N_2O. B) Reactive species formed in the pulse radiolysis of a solution of caged papain in water, formate and N_2O. Only species associated with the reactivity of the mixed disulfide at cysteine-25 are shown for clarity.

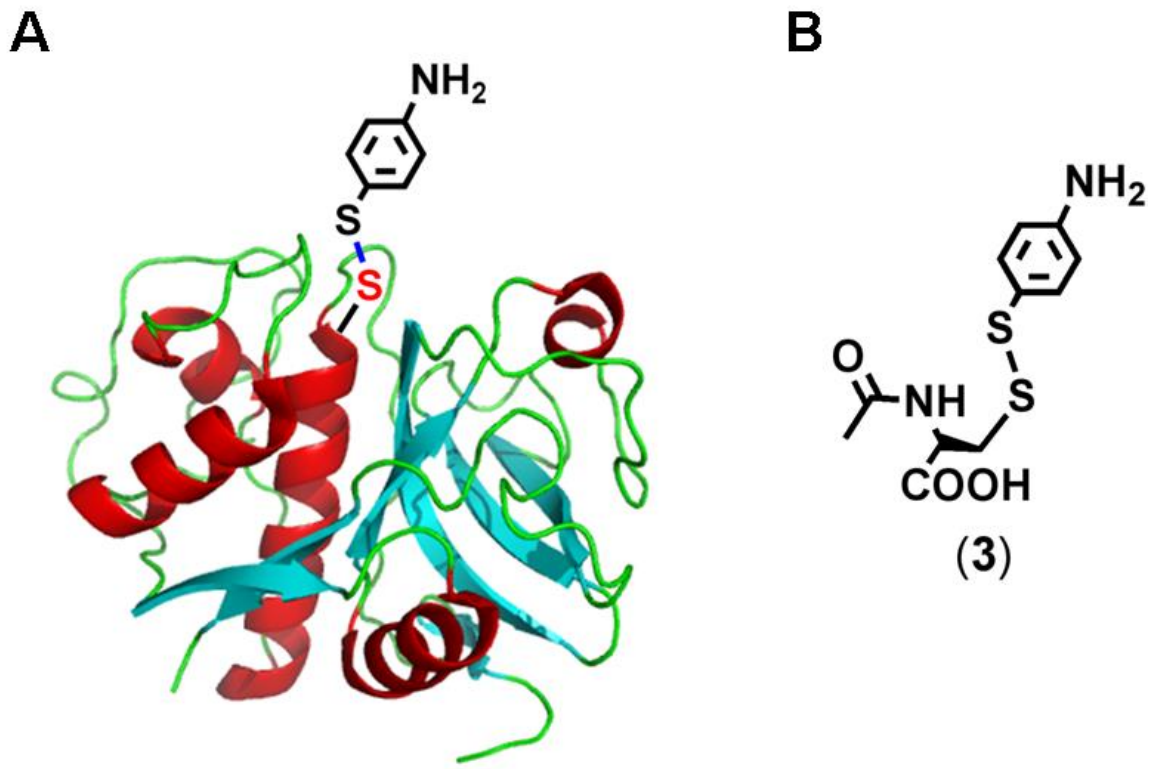

Figure 2. A)Crystal structure of papain from the latex of *Carica papaya* (pdb code 9PAP) illustrated using PyMOL [30]. The thiol of Cysteine 25 functionalised by the aromatic disulfide used in this work is also shown.B) The model compound used in this study.

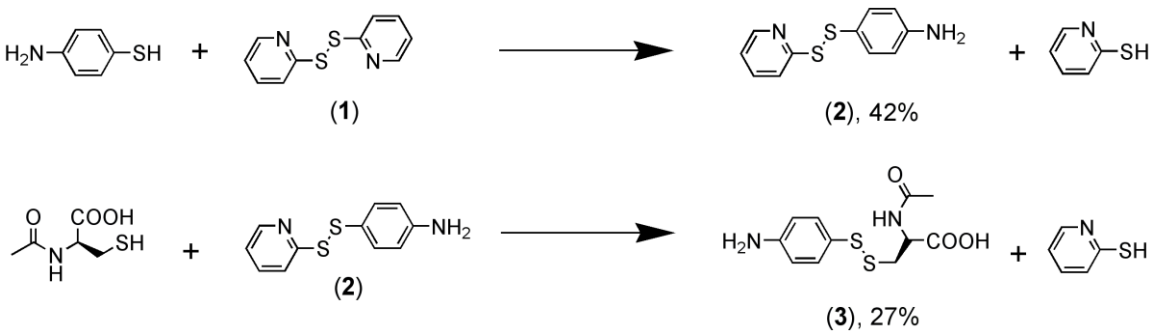

Figure 3. Synthesis of compound (**3**).

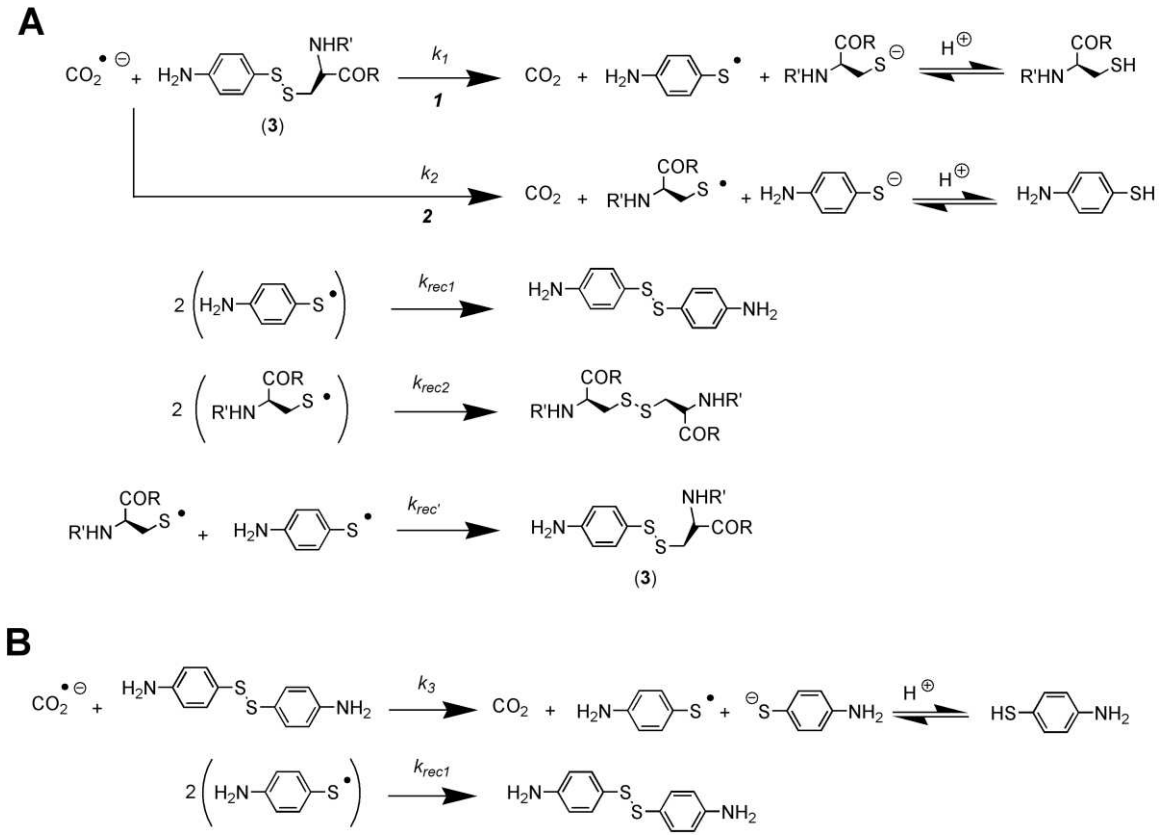

Figure 4. A)Reactions pathways for dissociation of (**3**) and subsequent recombination processes that were used in the kinetic model for the time resolved spectroscopic data shown in Fig. 5. R = OH and R′ = $COCH_3$. B) Reactions pathway for dissociation of bis(*p*-aminophenyl) disulfide which was used as kinetic model for the data shown in Fig. 5.

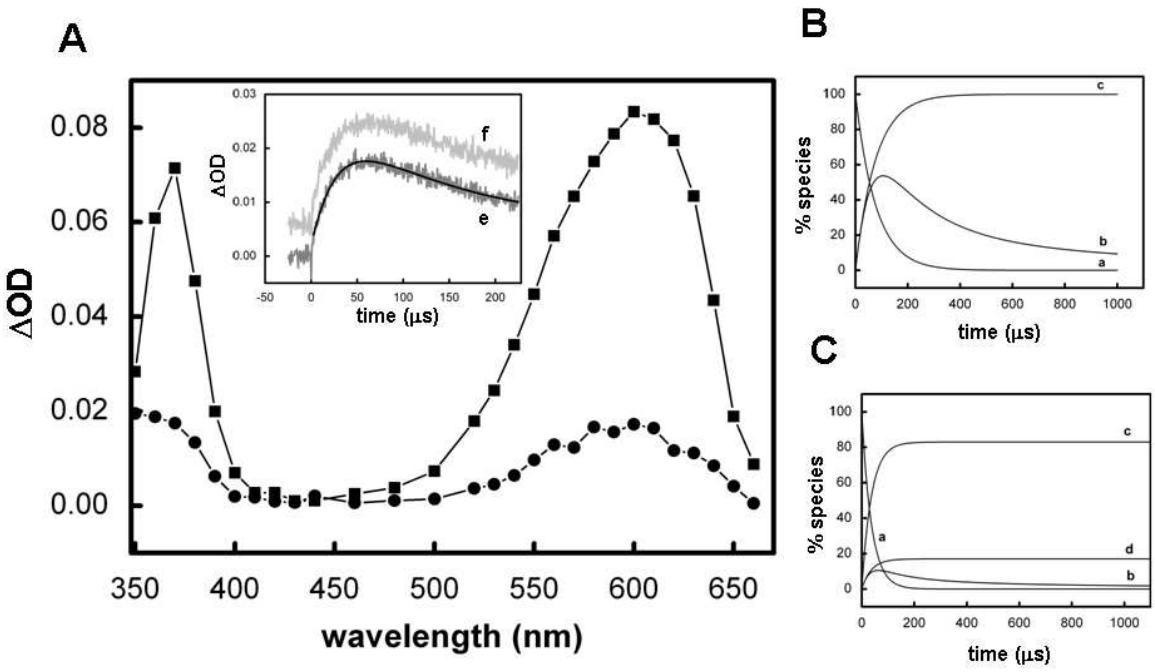

Figure 5. A). Transient absorption spectra recorded in pulse radiolysis of (**3**) in N_2O saturated 50 mM formate pH 6.2 (140 μM, -•-) and of *p*-aminophenyl disulfide (500 μM, -■-) in water: IPA (4:1) at 80 microseconds after the pulse (dose 7.3 Gray). The inset shows kinetic traces of (**3**) taken at 600 nm (**e**) and at 380 nm (**f**). The fit of the kinetic traces at 600 nm for (**3**) is also shown (black solid line). B) Time course of speciation profile after radiolysis of bis(*p*-aminophenyl) disulfide: disulfide radical anion (**a**), *p*-aminophenyl thiyl radical (**b**) and *p*-aminothiophenol (**c**). C) Time course of speciation profile after radiolysis of (**3**): disulfide radical anion (**a**), *p*-aminophenyl thiyl radical (**b**), *p*-aminothiophenol (**c**) and N-acetyl L-cysteine (**d**). For clarity, the speciation of the disulfide from radical recombination is omitted and in C) the speciation of the N-acetyl L-cysteine thiyl radical is omitted as well.

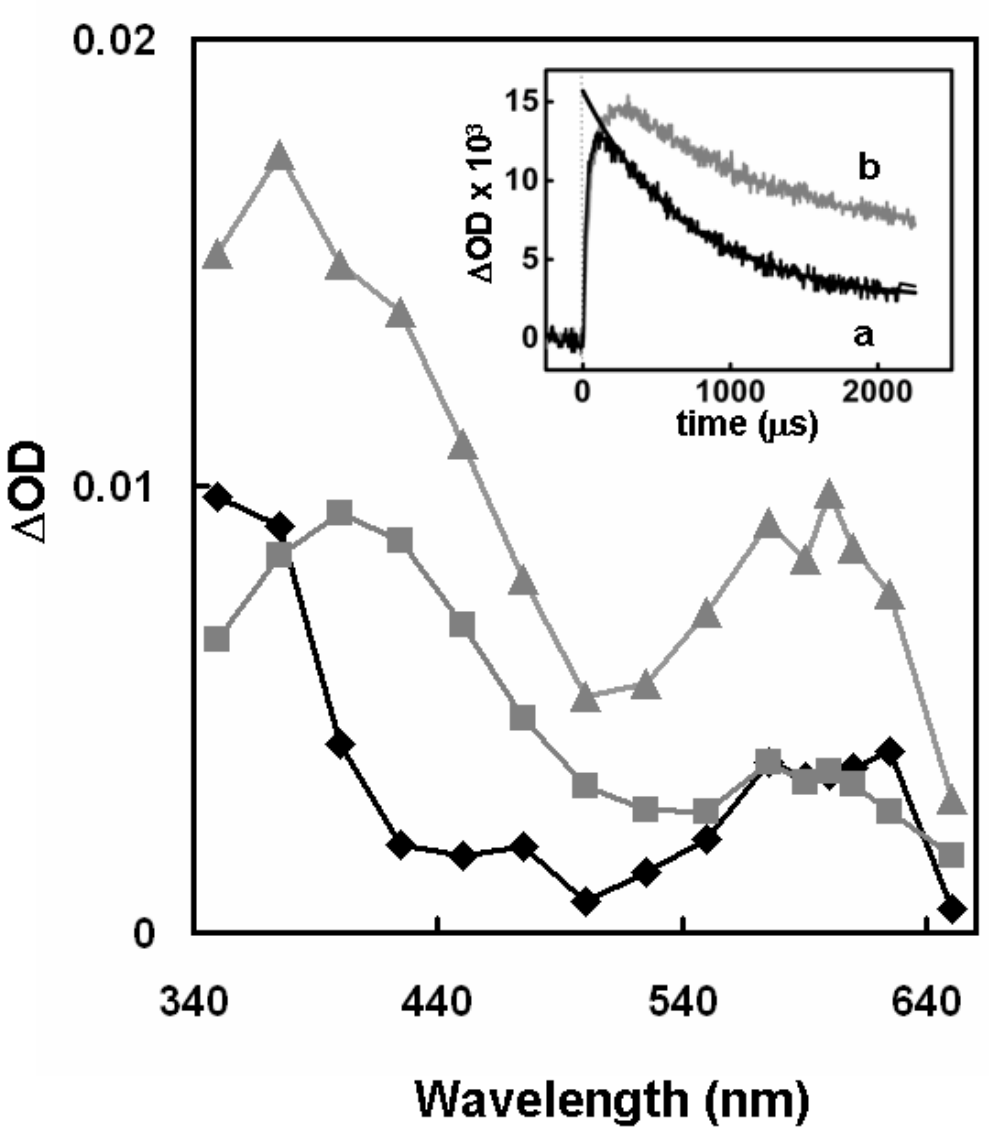

Figure 6. Transient absorption spectra recorded in pulse radiolysis of 12 μM solution of modified papain in 200 mM formate pH 6.2 at 5 (♦), 410 (▲) and 1610 (■) microseconds after the pulse (dose 19 Gray). The inset shows kinetic traces taken at 600 nm (a) and at 425 nm (b). The fitting of the 600 nm decay with the extrapolation to time zero is also shown.