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Thiol-disulfide exchange reactions occurring at modified bovine serum albumin detected using ellman's reagent (5, 5'-dithiobis (2-itrobenzoic acid)

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Abstract: Bovine serum albumin (BSA) is usually employed as a model protein because of being homologous with human serum albumin. Cysteine-34 of BSA has been oxidised with Ellman's reagent to produce BSA labelled with an Ellman's moiety (BSA-SE). The BSA-SE was then reacted with glutathione, N-acetylcysteine and D-penicillamine (D-pen). The two were able to release the Ellman's moiety bound at cysteine-34 while D-pen did not. Albumin labeled using Ellman's reagent was used to demonstrate the cleavage of a protein mixed disulphide. The kinetics of thiol disulfide interchange reactions involving formation of a chromophoric thiolate were determined by UV-visible spectroscopy. The reaction of thiolates with excess Ellman's reagent is used for quantitative estimation of thiol by measuring the absorption at λ, 412 nm. The disulfide exchange reactions occurring at Cys-34 of BSA was determined and the reduction of oxidized Cys-34 was studied in order to understand the reverse reaction. Spectroscopic evidence suggested that glutathione and N-acetylcysteine remove the label and produce BSA in a disulfide form. In contrast, D-pen reaction returned BSA to its thiolate form via mediation. It was observed that thio-disulfide exchange occurred at cysteine-34 labelled with Ellman's moiety. The implications to the redox status of plasma are discussed.

Keywords: Thiol-disulfide exchange, n-acetylcysteine, bovine serum albumin, d-penicillamine. sulfhydryl, glutathione, cysteine-34.

INTRODUCTION

In biological system sulfhydryl group are existing as in low molecular forms of homocysteine, glutathione and as well as in protein such as haemoglobin and albumin. From cows, bovine serum albumin basically a serum albumin can be derived. In laboratory experimental work this is usually used as standard of protein content. It is utilize to determine the amount or content of various other proteins when compare the unknown protein content with BSA known content. Sulfhydryl that -SH group is widely occurring in biological environment as smaller entities or molecules (Janatova, 1965). Albumin in blood with thiolate moiety at cysteine-34 serves as main protein with thiol (Turell *et al.,* 2013; Anraku *et al.,* 2013). The protein thiolate in systemic circulation contains sufficient amount of disulfide attached to glutathione as well as attached to cysteine or existing in form of sulfenic acid (Anraku *et al.,* 2013; Alvarez *et al.,* 2010). Serval clinical research work highlighted that the main assay carried out for disorder with oxidative stress focussed on cysteine-34 and protein with thiolates (Anraku *et al.,* 2013; Oettl and Marsche, 2010; Nagumo *et al.,* 2014). Some of the studies focused on cysteine effect on oxidative modification and other tried to find out the easy possible exchange of disulfide- thiol binding reversion at binding site (Christodoulou *et al.,* 2005; Lamprecht *et al.,* 2008; Turell *et al.,* 2008). Ellman's method is also used to check thiol amount resulting from reaction of Ellman's reagent in excess with thiolates by taking absorption of at lambda maximum of 412nm (Ellman *et al*., 1961; Wei *et al*., 2008; Carolina *et al*., 2006; Hanjing *et al*., 2012; Ridlles *et al*., 1983). In this study we have sought to briefly investigate this reaction where albumin labelled using Ellman's reagent has been prepared and used to demonstrate the cleavage of a protein mixed disulphide by the biological relevant thiolates species glutathione, N-acetylcysteine, D-penicillamine is the main objective of this current study.

MATERIALS AND METHODS

Materials

Reagents were purchased commercially. Ellman's reagent and Sephadex (G25 coarse) were purchased from Sigma Aldrich, St. Louis, Missouri United States). Bovine Serum Albumin (BSA >98% obtained by agarose gel electrophoresis lyophilised) (Sigma Aldrich, St. Louis, Missouri United States). U.V-visible, Unicam UV, 300 Spectrophotometer was also used in analysis of samples.

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Methods

Thiolate content determination in BSA

Ellman's reagent, stock solution of 10mL by dissolving 35.8mg of the reagent in phosphate buffer solution of pH 7.4. BSA stock solution of 50ml was prepared by dissolving 0.43mg in the same buffer solution. Solution of BSA in 8×ml was incorporated in sequence to various strengths of Ellman's reagent solutions of 0, 10, 20 30, 40, 50, 60, 80 and finally 90µl. this mixtures was kept for one hour to undergo through exchange reaction as shown in equation 1. Then spectra were recorded at 200 to 60nm of all the mixture after one hour reaction. Absorbances were plotted obtained at 412nm which is basically as function concentration of Ellman's reagent that showed Ellman ions maximum quantity obtained from samples of BSA to be tested. BSA thiolate amount was calculated from Beers Law at measuring wavelength of 412nm; ε = 14,150 $cm^{-1}M^{-1}$ on the basis of BSA molecular weight that is 66,000.

Preparation of Ellman's modified BSA (BSA-SSE)

BSA solution was prepared with 0.1g in 1ml of 0.1M phosphate buffer of pH 7.4 and Ellman's reagent solution was prepared by dissolving 1mg in 200µml of the same buffer. These solutions mixed and kept for 2 hours to react. The solution applied with care to column of 6cm×3cm packed with swollen Sephadex (G25 coarse). Phosphate buffer used for elution of this mixture. Protein present in eluent identified with trichloroacetic acid and collected. For complete elution of protein samples were periodically identified. Yellow band identification confirmed residual Ellman's reagent as well as anion and eluted with phosphate buffer. The amount of protein in solution was obtained with Beer Law at (λ_{max} = 280 nm; ε = 43,824 $cm^{-1}M^{-1}$) (Peters and Putman, 1975) and as it typically present from ~0.7 – 0.9 µmol per litre.

Treatment of isolated BSA-SE with thiolates

High excess of thiolates

Incubated protein solution aliquots of 2.5ml (74.5µmolL^{-1}) with thiolate to obtain 0.026 molL^{-1} solutions of respective thiolates that is N-acetylcysteine, glutathione and D-penicillamine. These solutions were left for duration of two hours for completion of reaction and analyzed spectrophotometrically at 250 to 600nm. Beer Law was used for calculation of released Ellman's anion at measuring wavelength λ_{max} of 412 nm: ε = 14,150$cm^{-1}M^{-1}$ (Riddles *et al.,* 2011).

Spectrophotometric analysis

Protein solution aliquot of 2.5ml equal to 74.5µmolL^{-1} was added into a cuvette to obtain spectrum. Thiolates; N-acetylcysteine, glutathione and D-penicillamine respective solutions of 50µl with final amount of 2.6 mmol L^{-1} were added with micropipette and the reaction was monitored with spectrophotometer at measuring wavelength of 412nm over a period of 30 minutes. These solutions were further kept for 90 minutes for completion of reaction and again recorded spectrophotometric analysis at 250 to 600nm for getting spectrum. Protein released Ellman's anion amount was obtained with Beer Law at λ_{max} = 412 nm, ε = 14,150 $cm^{-1}M^{-1}$ (Riddles *et al.,* 2011).

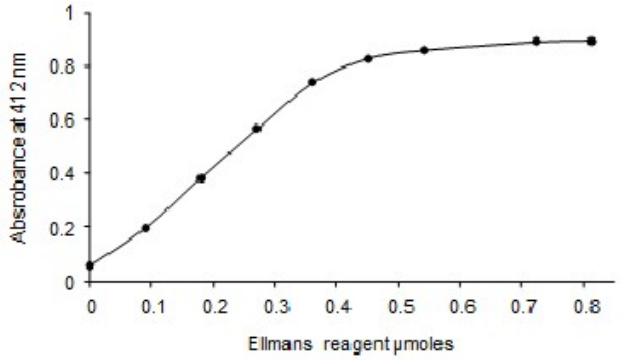

Fig. 1: The titration of BSA (3 ml of 0.86% in PBS; pH 7.4) with Ellman's reagent (0, 10, 20, 30, 40, 50, 60, 80 and 90 µL of 9.03mM). The intercept is consistent with the residual absorbance by BSA at 412 nm. The expected deviation from linear behaviour at high Ellman's reagent concentrations (~0.5µmol) marks the maximum amount of Ellman's anion that can be release for the given amount of BSA and is commensurate with the thiol status of the protein. Care must be exercised as the band edge associated with excess Ellman's reagent (λ_{max} 325nm) contributes to a small increase in the band at 412 nm at higher reagent concentrations (>0.55 µmol).

$$ESSE + RS^- \rightleftharpoons ESSR + ES^- \quad \text{-(1a)}$$

$$ESSE + 2RS^- \rightleftharpoons ESSR + ES^- + RS^- \rightleftharpoons RSSR + 2ES^- \quad \text{-(1b)}$$

$$BSA + ESSE \rightleftharpoons BSA\text{-}SSE + ES^- \quad \text{-(2)}$$

$$BSA\text{-}SE + RS^- \rightleftharpoons BSA\text{-}SR + ES^- \quad \text{-(3)}$$

$$BSA + RSSR \rightleftharpoons BSA\text{-}SR + RS^- \quad \text{-(4)}$$

Scheme 1: The thiol disulfide equilibria relevant to this study

BSA-SE released in-situ treatment with thiolates

BSA stock solution 0.22g of 25ml as well as Ellman's reagent 15.1mg in 5ml in 0.1M phosphate buffer of pH 7.4 was obtained. Protein solutions three aliquots of 0.40µmoles with thiolate cysteine of 0.172 µmoles were taken in sample bottles and added Ellman's reagent of 20 µL (0.15 µmoles) and placed in dark for 24 hour till completion of reaction. Simultaneously with above protein fourth sample of 3ml was added into cuvette for spectrophotometric analysis at 250 to 600nm. This sample was also added Ellman's reagent and checked the reaction at 412nm spectrophotometrically over two hours. Again sample was placed a side and recorded spectrum after 22 hours. The above prepared equilibrium solution were

added to cuvette and taken spectra at 200 to 60nm. These solutions were checked for 5 minutes at 412nm and then treated separately with 1.54µmoles of glutathione, D penicillamine 1.57µmoles or N-acetylcysteine of 1.85µmoles and for two hours monitored at 412nm. These were kept in dark place over 22 hours to complete reaction and analyzed for spectra at 200 to 600nm. In order to determine Ellman's reagent and glutathione reaction rate a control study was performed. In phosphate buffer of 3ml was added Ellman's solution of 20 µL (0.15 µmoles). For five minutes this solution was monitored at 412nm and at same time treated with 20 µL 1.5 µmoles 10x excess solution of glutathione and monitored the reaction for more 15 minutes.

RESULTS

The Ellman reagent was released as increasing concentration of BSA gave optimum release of Ellman's anion with compensation of BSA natural absorbance at 412nm as given in fig. 1 and obtained the correct value of status of thiolate.

In this assay Ellman's anion obtained but also produced and also generated BSA labelled with Ellman's moiety at site of cysteine-34 as shown in equation 2. It created capability of more BSA attached with Ellman moiety at cysteine-34. When BSA incubated with sufficient of Ellman's reagent for overnight which was done on basis of thiolate assay is shown in fig. 1 then proceeded by separation with chromatographic with Sephadex® G25 resultant in BSA modified by Ellman reagent in phosphate buffer solution. The solution were analyzed at 280nm helped in molar absorptivity (ε = 43,824 $cm^{-1}M^{-1}$) and protein was calculated from eluent (Peters and Putman, 1975) of the solution at 280 nm it is possible to give the protein concentration directly from eluent. It was desired to observe that GSH, N-acetylcysteine and D-penicillamine being small thiolates reaction with cysteine-34 in form of disulphide so taken eluent rich of protein. It was assumed that maximum concentration of anion might be obtained from modified protein as eluent with 10 fold amount of thiolate was used in fig. 2. The thiolate produced Ellman's anion as was desired from solution of protein of 74.5µmol L^{-1} that was efficiently 32.0µmol L^{-1} BSA-SE. GSH as well as N-acetylcysteine both produced Ellman's anion (106µmolL^{-1}) but 67µmol L^{-1} Ellman's anion resulted by D-penicillamine. As the values obtained was exceeding the assumed amount proportionate the exchange of modified protein as BSA-SE with thiolate. Experiments were conducted carefully in chromatographic analysis to isolate modified protein Ellman's reagent in surplus as showed by spectra of BSA-SE in spectrophotometric analysis gave rise band at 325nm which showed presence of Ellman's reagent in sample given in fig. 2.

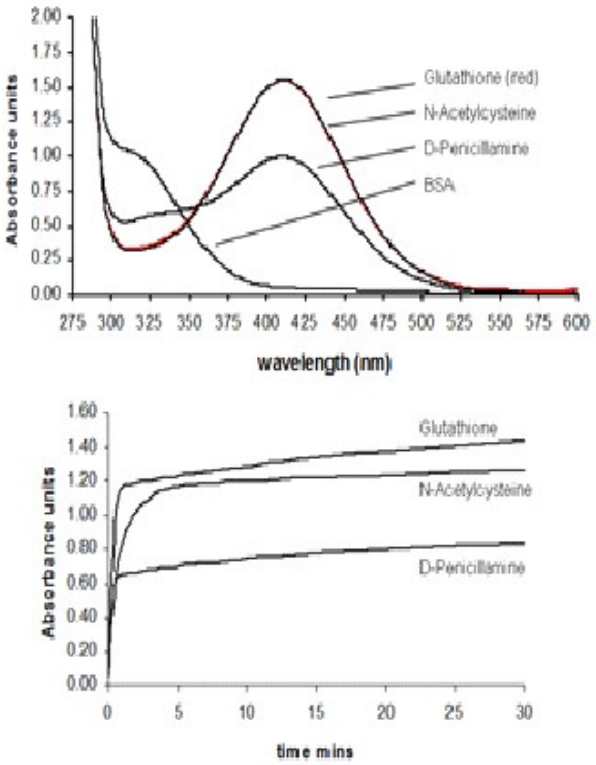

Fig. 2: Left: Treatment of chromatographically isolated BSA-SE (75µmol L^{-1}) with thiolate (glutathione (red), N-acetylcysteine, D-penicillamine 0.026 mol L^{-1}): samples incubated for 2hr. The formation of Ellman's anion (equation 2) is evident from the appearance of a band at 412nm. The distinct shoulder at λ_{max} 325nm in the spectrum of the BSA solution is indicative of adsorbed Ellman's reagent. Right: The change in absorbance over time (λ_{max} 412nm, 0-30 mins) when chromatographically isolated BSA-SE (75µmol L^{-1}) is treated with thiolate (glutathione, N-acetylcysteine, D-penicillamine at 2.6 mmol L^{-1}). The slow increase in Ellman's anion concentration after 2 mins supports the opinion that there are two pools of Ellman's species on the protein i.e. adsorbed Ellman's reagent (rapid) and BSA-SE (slow).

To know disulphide state of BSA exchange behavior, it was clear from concentration of Ellman reagent added to mixture could be controlled to subdue the anion generation by exchange reaction of simple thiol-disulphide reaction as in equation 1a from excess Ellman's reagent. Rather surplus amount of Ellman's reagent interfered the equilibrium in equation 2 to the right side and specified stoichiometric close amount was used as to achieve equilibrium after 24 hours. As it was aimed to investigate a reaction with quasi-equilibrium and again considered for single time point analysis with 2hour monitoring of reaction and again analysed after 24 hour to know dynamic of the reaction. In this reaction state BSA and Ellman's reagent reacted and release of Ellman's anion was observed slowly as shown in fig. 3 at t½ = 40 mins. As it was not surprising that cysteine-34 existed

with protein and the exchange of thiol-disulphide reaction to be slow obvious in fig. 3 GSH/ESSE. Due reduce amount of Ellman's reagent and less reagent push the equilibrium to right side in equation 2. As mixture left for overnight and lead to quasi equilibrium. UV-spectrophotometric analysis was done to attain Ellman's anion as indicated in fig. 3.

The equilibrium mixture was divided into portions of 3ml each for treatment with thiolates of different ranges and as the previous reaction was of slow nature and then excess of thiolate was used to obtain Ellman's moiety with cysteine-34 as shown in fig. 3. The mixtures treated with thiolate were found again showed biphasic behaviour in time course. The Ellman's anion obtained rapidly initially just after two minutes. As fig. 3 shows the consistent rapid increase in reaction of thiolate and residual Ellman's reagent in mixture. It is also observed that rapid increase shown for glutathione as well as N-acetylcysteine and half for penicillamine. When Elman's reagent pool depleted the Ellman's anion of mixtures treated with glutathione and N-acetylcysteine clear in fig. 3. The reaction again became slow down. After 24 hour, Ellman's anion was consistently completed and the Ellman's species converted into Ellman's anion shown in scheme 1. It is noted that glutathione as well N-acetylcysteine detached Ellman's label from protein as the protein found oxidized in form of disulfide either with glutathione of N-acetylcysteine as given in equation 3.

The penicillamine reaction profile was interesting. As the Ellman's anion expectedly increase was observed in release as exchanged occurred of penicillamine with free Ellman's reagent but after that concentration of Ellman's anion gradually decreased and after 75 minutes it was noted very low as compared to initial exchange. Ellman's anion amount after 24 hours was 17.0µmol L^{-1} that was 35% of initial amount. Initially D-penicillamine reacted with Ellman's reagent that was in excess, further Ellman's reagent was consumed instead to generate in this reaction and scheme 1 shows several components in the mixture. As previous work shows that D-penicillamine has less exchange capacity rather than glutathione as well as N-acetylcysteine and here it is described that D-penicillamine located very close to left as shown in equation 3.

DISCUSSION

Commercially BSA in thiolate form contains ~30 - 50% of protein which is typically available as value of protein varies in different batches so its content information is very important. Thiolate in BSA solution could be titrated with Ellman's reagent. Spectrophotometrically Ellman reagent release can be evaluated at measuring wavelength of 412nm; ε = 14,150 $cm^{-1}M^{-1}$ (Riddles *et al.,* 2011) as elaborated by fig. 1. Wide range of binding capability of albumin observed with variety of organic drug such as salicylates and benzoates (Tabachnick and Korcek, 2014; Nishijo *et al.,* 2009). As Ellman's reagent is being a benzoic acid and reflected as not new that unreacted Ellman's reagent bound with protein and passed through column as high molecular weight portion. The inconsistency between observations got for glutathione, N-acetylcysteine and D-penicillamine as shown in fig. 2. Depending on the resultant thiol-disulphide equilibrium achieved. Glutathione as well N-acetylcysteine contributed in 2nd equilibrium in equation 1b lead to generation of two equivalents of Ellman's anion and homo-sulphide as respective GSSG and NAC_2. While D-penicillamine only contributed in 1st equilibrium and resulted in only one equivalent Ellman's anion (Ullah *et al.,* 2014). The calculated amount was noted to be 4.8mmolL^{-1} ES^-. It might be elaborated that 85% of protein cysteine-34 with Ellman's moiety. As equilibrium established in the way small amount of unmodified ESSE is shown at 325nm in spectrophotometric analysis. Reaction of simple thiolate of BSA with Ellman's reagent as equation 2 showed the equilibrium position in mixed way in disulphide state (Whitesides *et al.,* 2007: Wilson *et al.,* 2017).

Protein was having a sufficient Ellman's reagent pool and reacted rapidly thiolate showed difficulties for determining Ellman's moiety bound with cysteine-34 reactivity. It was repeated at low amount of thiolate 2.6mmolL^{-1} followed by reaction over time can be observed from two phase pattern reaction in fig. 2. Initially a dominant reaction occurred rapidly showed adsorbed ESSE interaction with thiolate in equation 1(Whitesides *et al.,* 2007: Wilson *et al.,* 2017) and 2nd reaction of slow one showed possible Ellman's moiety with protein in equation 3. As excess of adsorbed Ellman's reagent pool as well as complex nature of exchange kinetic resulted from equation 1 to 3 might lead to be concluded for further testing. As the obtained data showed that when D-penicillamine reacted with excess Ellman's reagent gave large amount of Ellman's anion in equation 1a as excess of Ellman's anion affected the equilibrium location of the equation 2 and shifted it to the left. Furthermore, in solution D-penicillamine was unable for reacting with BSA species in direct way because of steric issues but it is able to react slowly and supplying Ellman's reagent steady in equation 2. The establishment of a dynamic equilibrium allowed D-penicillamine and acted in such way that reduced the protein instead of direct interacting.

CONCLUSION

It was observed in this research work that thio-disulfide exchange occurred at cysteine-34 labelled with Ellman's moiety. As to demonstrate albumin mixed disulfide reactivity in simple way is very rare. The reported studies

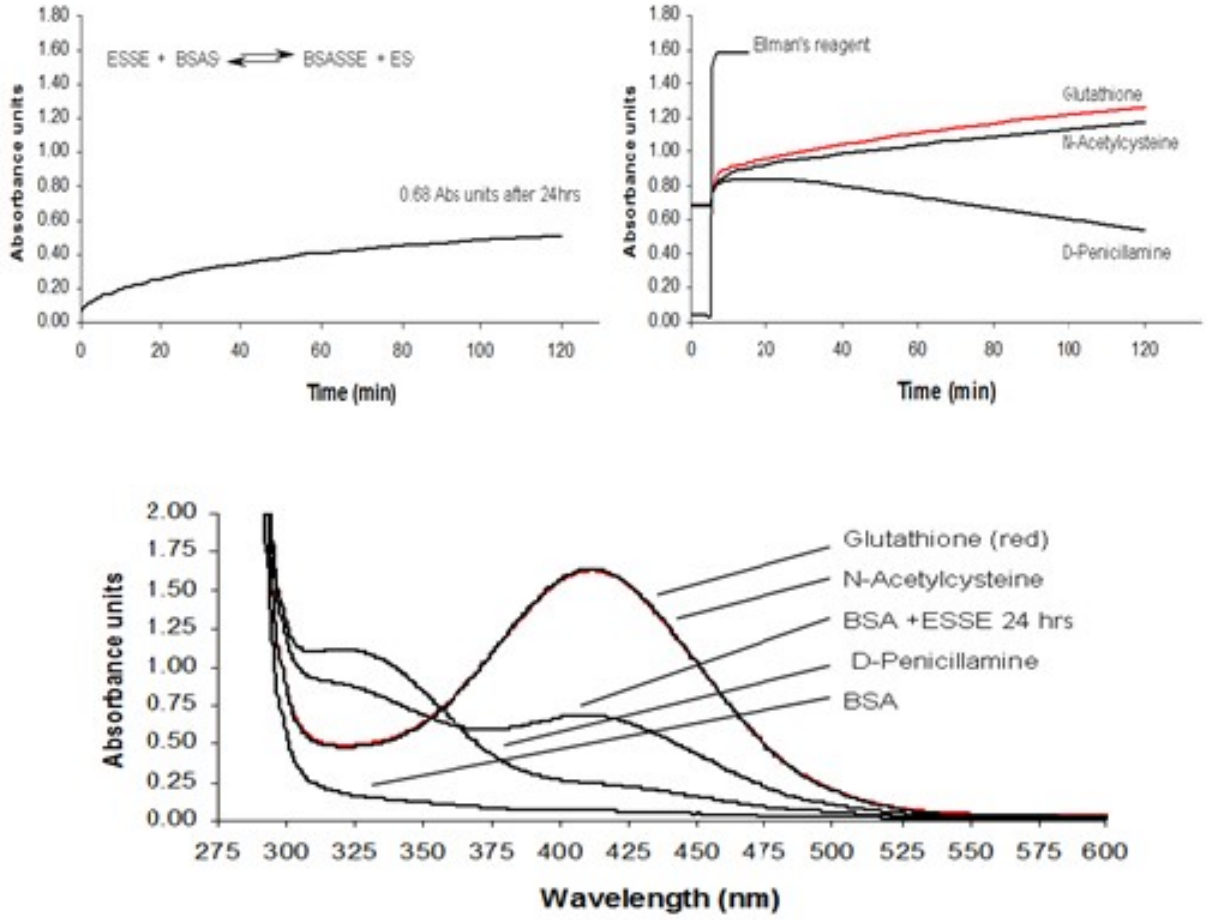

Fig. 3: Top left: The reaction of BSA (57.3µmol L^{-1} thiol form) with Ellman's reagent (50.0µmol L^{-1}) monitored at 412 nm over a 2 hr period. The sample was placed in the dark and analysis recommencing the next day. Top right: Two datum points were collected prior to the addition of thiolate after which the solution was monitored for a second 2 hr period. The reaction of Ellman's reagent (50µmol L^{-1}) with glutathione (0.51 mmol L^{-1}; no BSA) was carried out under similar conditions to allow a visual comparison of the reactivity of free thiolate with that found in BSA and demonstrate the maximum amount of Ellman's anion which can be generated in solution. Bottom. Representative spectra of the solutions at the key time points in the reaction *viz* the BSA starting solution, the formation of BSA-SE, the reaction of BSA-SE with thiolates (glutathione, N-Acetylcysteine and D-penicillamine). The formation of Ellman's anion (equation 2) is evident from the appearance of a band at 412nm. The distinct shoulder at λ_{max} 325 nm in the spectrum of the BSA solution and the penicillamine treated solution is indicative of the presence of Ellman's reagent in the mixture.

did not give an idea about rate and nature of complex formed as result of reaction. It was expected that these reactions might be slow but surprised by obtaining very short half-life of 40 minutes. It was taken as study of full kinetic nature but can't able to get modified protein in form of BAS-SE that would free form attached Ellman's anion but were aware of the fact that obtained correct instantaneous rates of the whole processes and might be more visible results with use of more qualitative approach. The measurement of thiolate status of albumin is not uncommon in clinical analysis. However, what is surprising here is that the reactions take some hours to come to completion. While the use of excess Ellman's reagent will accelerate the reaction to its equilibrium position it would seem that Ellman's modified proteins continue to be reactive towards thiolates such as glutathione and N-acetylcysteine. Furthermore, it has long been accepted that D-penicillamine has a different medicinal chemistry to glutathione and that glutathione is the more reactive of these two species. What is surprising in this study is that the enhanced reactivity of glutathione leaves the protein in an oxidised form whereas the more limited nature of D-penicillamine, by hindering the formation of protein-disulphide acts to reduce the protein, all-be-it through chemical redox intermediaries (ES-, ESSE). This behaviour may have some relevance to the efficacy of these species.

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