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The reactivity of complexed thiolates with Ellman's reagent: An NMR spectroscopic study ☆

Naseem Ullah [a], Muhammad Mukhitar [a], Muhammad Farid Khan [a,*], John Reglinski [b,*]

[a] Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Gomal University, D.I. Khan, KPK, Pakistan
[b] Department of Pure & Applied Chemistry, University of Strathclyde, 295 Cathedral St., Glasgow G1 1XL, UK

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ABSTRACT

The Ellman's based thiol–disulfide exchange process used to quantify sulfhydryl groups in biology and medicine is interrogated using ^{1}H NMR methods. Although not as accurate as the spectrophotometric assay NMR spectroscopy has value for the study of complex mixtures as it can identify all the key species in the mixture including the mixed disulfides of Ellman's reagent and its sulfenic acid. We have used NMR to analysis complex mixtures involving metal (arsenic, lead, palladium) thiolates where we are able to show that Ellman's reagent can exchange with coordinate thiolate but at the expense of the accuracy of the analysis.

1. Introduction

Ellman's reagent (5,5′-dithiobis(2-nitrobenzoic acid)) has been a important reagent for the spectrophotometric assay of sulfhydryl groups in biology for many years [1–3]. The accuracy of the analysis is based on controlling the two related equilibria (Eq. (1)) which form when treating Ellman's reagent (ESSE) with thiolate (RS^-). Care is usually taken to avoid the involvement of the second exchange reaction (and hence the reaction of thiolate, RS^- with ESSR) and consequently the assay is typically conducted using an excess of Ellman's reagent. However, if Ellman's reagent is present at too high a concentration during the analysis, the deconvolution of the bands in the spectra (ESSE, λ_{max} 325 nm: ES^-, λ_{max} 412 nm) becomes difficult and the accuracy of the analysis is compromised. The solubility of Ellman's reagent and the pK_a of the sulfhydryl groups also requires some attention. Ellman's reagent is only sparingly soluble in water below a pH of 7. Furthermore, if the pH rises above 8, the solution develops an intense colouration which cannot be reversed [4]. Fortuitously, the important thiolate species typically studied (e.g. glutathione, human serum albumin) have an appropriate pK_a (~8.5) which makes analysis at physiological pH possible.

$$ESSE + 2RS^- \overset{K_1}{\Longleftrightarrow} ESSR + RS^- + ES^- \overset{K_2}{\Longleftrightarrow} RSSR + 2ES^- \quad (1)$$

The application of the spectrophotometric Ellman's assay to more complex mixtures, specifically where there is the potential for competition for the sulfhydryl groups, can be problematic. This situation arises with the speciation of metals in biological mixtures, where there is the possibility of dynamic exchange between species bound to the sulfhydryl groups with the parent ligand, solvent (water) or buffer [5]. In these instances there is concern that the Ellman's reagent can perturb the equilibrium and compete for the coordinated thiolate as well as the "free" sulfhydryl present in solution. The consequences of this will be an over-estimation of the amount of free sulfhydryl present in the system.

We are interested in the behaviour of heavy metals at sulfhydryl groups in biological mixtures and have elected to study thiolate complexes of trivalent arsenic and divalent palladium and lead in competitive studies with Ellman's reagent. The former was chosen as it forms a tris-monodentate species ($As(SR)_3$) where the arsenic is directly bonded to each thiolate via their respective sulfur [6–8]. Palladium and lead were chosen as they are known to form a range of chelate complexes with biologically relevant thiolates [9–12]. For these metals the coordination sphere of the metal is made up from a sulfur and a nitrogen from the ligands. In an attempt to understand the species in solutions we have elected to conduct this study using NMR methods (c.f. spectrophotometric methods) as these allow us to identify additional components of the reaction mixture and their relative abundance.

☆ This work was conducted at Strathclyde University. All authors contributed equally to this study.

* Corresponding authors. Tel.: +44 141 548 2349; fax: +44 141 548 4822 (J. Reglinski).

E-mail addresses: drfarid_2006@yahoo.com (M.F. Khan), j.reglinski@strath.ac.uk (J. Reglinski).

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2. Materials and methods

All reagents were commercially obtained. Ellman's reagent and glutathione (97%) were purchased from Sigma Aldrich. ^{1}H NMR spectra were obtained using a Bruker AVANCE 3 spectrometer operating at 400.12 MHz. Samples were maintained at 300 K during spectral acquisition. The free induction decay was generated by a 3.13 μs pulse width corresponding to a 30° pulse with a 2 s delay between pulses. Each data set was collected in 32 k of memory. A 1 Hz line broadening function was applied before Fourier transformation to reduce the effect of the baseline noise.

2.1. NMR assignments for Ellman's reagent, Ellman's anion and the thiolate mixed disulfides of Ellman's reagent

2.1.1. N-Acetylcysteine

Ellman's reagent (10 mg, 25.2 μmol) was dissolved in 1.5 ml of buffer (0.1 M KH_2PO_4 in ^2H_2O at pH 7.4). 300 μL of this solution were placed in an NMR tube (0.5 mm diameter). Solutions (500 μl) containing 0.4 mg (2.48 μmol), 0.8 mg (4.96 μmol), 1.2 mg (7.44 μmol), 1.6 mg (9.92 μmol)and 2.0 mg (12.40 μmol) of *N*-acetylcysteine were prepared. These amounts were added to a series of NMR tubes containing the Ellman's reagent. The solutions were mixed and the NMR spectra recorded. An additional sample of Ellman's reagent 1 mg in 800 μL was prepared as a reference sample.

2.1.2. D-Penicillamine

Ellman's reagent (10 mg, 25.2 μmol) was dissolved in 1.5 ml of buffer (0.1 M KH_2PO_4 in ^2H_2O at pH 7.4). 300 μL (5 μmol) of this solution were placed in an NMR tube (0.5 mm diameter). Solutions (500 μL) containing 0.19 mg (1.27 μmol), 0.38 mg (2.54 μmol), 0.57 mg (3.81 μmol), 0.76 mg (5.08 μmol) and 1.13 mg (7.57 μmol) of D-penicillamine were prepared. These amounts were added to a series of NMR tubes containing the Ellman's reagent. The solutions were mixed and the NMR spectra recorded.

2.2. Calibration of the thiol disulfide exchange by NMR methods

Stock solutions of Ellman's reagent (74.4 mg/10 mL) and glutathione (0.292 g/5 mL) were prepared using 0.1 M KH_2PO_4 in ^2H_2O at pH 7.4. 500 μL aliquots of the Ellmans solution (9.38 μmol) were placed in six 0.5 mm diameter NMR tube. To these six NMR tubes were added 10, 20, 30, 40, 50 and 60 μL of the glutathione solution (1.70 μmol/10 μL). The samples were mixed their ^{1}H NMR spectra recorded. An additional sample of Ellman's reagent 1 mg in 800 μL was prepared as a reference sample. The spectra obtained were processed as described above. The integrals of the key resonances were used to calculate the relative amount of Ellman's reagent, Ellman's anion and the glutathione–Ellman's mixed disulfide present in the samples.

2.3. Alkaline hydrolysis of Ellman's reagent

1.0 mg of Ellman's reagent was treated with 0.8 ml of 0.1 M NaOD in a 0.5 mm diameter NMR tube and the ^{1}H NMR spectrum recorded. The resulting solution was treated with 4 μL of 20 vol. H_2O_2. The solution was allowed to react overnight in the tube (loosely fitted cap) after which the spectrum was re-recorded.

2.4. Interaction of Ellman's reagent with thio-arsinites

A series of tris-thiolato-arsenite (glutathione, D-penicillamine, *N*-acetylcysteine) solutions (300 μL) were prepared *in situ* using 0.1 M KH_2PO_4 in ^2H_2O at pH 7.4 using sodium arsenite and three equivalents of the corresponding thiol. These were added to a solution of Ellman's reagent (2 mg, 5.0 μmol) also dissolved in 300 μL, 0.1 M KH_2PO_4 in ^2H_2O at pH 7.4.

Amounts	$As(SG)_3$:	0.42, 0.86, 1.28, 1.70, 2.12, 2.56, 2.96, 3.40 μmol
	$As(NAC)_3$:	0.42, 0.86, 1.28, 1.70 μmol
	$As(PEN)_3$:	0.42, 0.86, 1.28, 1.70, 2.56, 3.40 μmol

2.5. Interaction of Ellman's reagent with Lead thiolates ($Pb(SR)_2$)

A series of bis-thiolato-lead (glutathione, D-penicillamine, *N*-acetylcysteine) solutions (300 μL) were prepared *in situ* using 0.1 M KH_2PO_4 in ^2H_2O at pH 7.4 using lead acetate and two equivalents of the corresponding thiol. These solutions (0.63, 1.26, 1.90, 2.53 and 3.16 μmol Pb) were added to a solution of Ellman's reagent (2 mg, 5.0 μmol) also dissolved in 300 μL, 0.1 M KH_2PO_4 in ^2H_2O at pH 7.4.

2.6. Interaction of Ellman's reagent with palladium thiolates

A series of mono and bis-thiolato-palladium (glutathione, D-penicillamine, *N*-acetylcysteine) solutions (300 μL) were prepared *in situ* using 0.1 M KH_2PO_4 in ^2H_2O at pH 7.4 using palladium nitrate dihydrate and either one or two equivalents of the corresponding thiolate. These solutions (1.27, 2.53, 3.80, 5.07, 6.33 and 7.60 μmol Pd) were added to a solution of Ellman's reagent (2 mg, 5.0 μmol) also dissolved in 300 μL, 0.1 M KH_2PO_4 in ^2H_2O at pH 7.4.

2.7. Spectrophotometric analysis of the effect of heavy metals on the Ellman's calibration chart

A standard Ellman's spectrophotometric assay was conducted using Ellman's reagents (100 μmol L^{-1} final concentration, 0.1 M KH_2PO_4 at pH 7.4) and a range of *N*-acetylcysteine solutions (20–100 μmol L^{-1}) in the absence and presence of arsenic, lead and palladium each at 100 μmol L^{-1}.

3. Results and discussion

The NMR analysis of solutions generated by titrating thiolate; *N*-acetylcysteine (NAC, Fig. 1) reduced glutathione (GSH) and D-penicillamine (PEN) into Ellman's reagent clearly allows the identification of Ellman's anion (ES^-), Ellman's reagent (ESSE) and the mixed disulfide (ESSR) in the ^{1}H NMR spectra. The resonances from Ellman's anion appear to higher field due to the effect of the distributed charge on the ring protons (Fig. 1, Table 1). As expected the resonances from the reagent itself and the mixed disulfide appear in a similar region of the spectrum. There is a slight overlap of certain resonances from each Ellman's based species but, in the main, the assigned resonances are distinct and can be used to calculate the relative concentration of the three species in solution. Thus a calibration curve can be generated by titrating a thiolate solution into a solution of Ellman's reagent. By plotting the relative integrals of the resonance assigned to Ellman's anion against the amount of thiolate used we obtain a graph (Fig. 2) which is linear and which is consistent with what is observed spectrophotometrically. The intrinsic error in the integrals derived from NMR spectra is much higher than those derived from optical methods and it is unlikely that NMR will replace spectrophotometry for quantitative measurements. The mixed disulfide (ESSR, R = thiol) and homoleptic disulfides (RSSR) are also dominant features in the high field region (δ 4.2–δ 2.0) of the spectra (Fig. 1) making it possible to simultaneously observe the five components (ES^-, ESSE, ESSR, RSSR and RSH) of the complex