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Direct measurement of the equilibrium between glutathione and dithiothreitol by high performance liquid chromatography

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The equilibrium constant between reduced glutathione (GSH), oxidized glutathione (GSSG), reduced dithiothreitol (DTT^{SH}_{SH}), and oxidized dithiothreitol (DTT^{S}_{S}) has been directly measured by high performance liquid chromatography analysis of equilibrium mixtures The equilibrium constant at 25°C for the reaction GSSG + $DTT^{SH}_{SH} \rightleftharpoons$ 2GSH + DTT^{S}_{S} varies from approximately 200 M, below pH 8, to approximately 2800 M, above pH 11 The observed pH dependence is generally consistent with published values of acid dissociation constants of these thiols

Glutathione, Dithiothreitol, Thiol reagent, Disulfide exchange

1. INTRODUCTION

Dithiothreitol and glutathione are common reagents for the study of disulfide exchange reactions of protein disulfides [1–9] The stability of a disulfide bond in a protein can be represented by the equilibrium constant of the thiol–disulfide exchange between that protein disulfide and a reference thiol species such as GSH and DTT It is sometimes useful to measure the stability of a disulfide bond by using both GSH and DTT. In this paper, we report results on a direct measurement of this equilibrium between glutathione and dithiothreitol

$$\text{GSSG} + \text{DTT}^{SH}_{SH} \underset{}{\overset{K_{SH}}{\rightleftharpoons}} 2\ \text{GSH} + \text{DTT}^{S}_{S} \qquad (1)$$

Several values of this equilibrium constant have been published. Cleland [10] reported a value of 1.3×10^4 M for the reduction of cystine by DTT at pH 7.0. Szajewski and Whitesides [11] report a value of 8.8×10^1 M at 30°C and pH 7.0, from equilibrium measurements Creighton [12] has reported a value of $1\ 16 \times 10^3$ M at 25°C and pH 8 7, measured indirectly from the equilibrium constants of disulfide exchange between these reagents and disulfide bonds in BPTI.

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Abbreviations GSH, reduced glutathione, GSSG, oxidized glutathione, DTT or DTT^{SH}_{SH}, reduced dithiothreitol, DTT^{S}_{S}, oxidized dithiothreitol, TFA, trifluoroacetic acid, DTNB, Ellman's reagent [5',5'-dithio*bis*(2-nitrobenzoic acid)]; HPLC, high performance liquid chromatography, BPTI, bovine pancreatic trypsin inhibitor

We are currently carrying out equilibrium studies of disulfide exchange of a protein using both dithiothreitol and glutathione Surprisingly, the equilibrium constant between dithiothreitol and glutathione obtained indirectly in our studies, approximately 120 M at pH 7 0 (unpublished data), was found to be different from previously reported values. In order to resolve this puzzle, we have measured the equilibrium constant between dithiothreitol and glutathione (Eqn (1)) directly by HPLC methods between pH 6 and 12.

2. MATERIALS AND METHODS

GSH and GSSG were obtained from both Sigma and Amresco Research Company DTT^{SH}_{SH} and DTT^{S}_{S} were obtained from Aldrich DTT^{S}_{S} was purified to eliminate strong oxidizing agents by a procedure described by Creighton [13] TFA (HPLC Spectra Grade) and DTNB were obtained from Pierce Chemical Company

Since all concentrations were measured from HPLC peak areas, the correlations between peak area and concentration were determined for each species The concentrations of stock solutions of each molecule were determined spectroscopically with minimum exposure to air The concentration of GSSG solution at pH 7 was determined by measuring the absorbance at 248 nm using an extinction coefficient of 383 ± 8 $M^{-1}\,cm^{-1}$ [7] The concentration of DTT^{S}_{S} at pH 8 was determined by measuring the absorbance at 283 nm and 310 nm and using extinction coefficients of 273 and 110 $M^{-1}\,cm^{-1}$ respectively [14] The concentrations of DTT^{SH}_{SH} and GSH solutions at pH 7 were determined by using DTNB and an extinction coefficient of 14 150 $M^{-1}\,cm^{-1}$ for the 2-nitro-5-thiobenzoate dianion at 412 nm [15]

All solutions were acidified to pH 2 with 6 M HCl before injecting into the HPLC The relationship between peak area and concentration is described by the equation $C = Q(A/V)$ where C is the concentration of the solution in mM, Q is the response factor, A is the peak area in AU-sec, and V is the injection volume in μl The response factors were found to be 13 9 for GSSG, 42 7 for GSH, 76 1 for DTT^{S}_{S}, and 103 2 for DTT^{SH}_{SH} The relationships of area to injection volume were linear up to an area of 14 AU-sec, corresponding to a peak

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absorbance of approximately 0 75 The correlation coefficient, R, was greater than 0 999 in each case

In order to avoid air oxidation of the thiol groups, the buffers used to prepare all solutions were degassed by alternately evacuating air from the vials and purging with argon for at least 30 min All reaction vials were kept under a positive pressure of argon during the reaction Buffers contained both 0 1 M Tris-HCl and 0 05 M potassium phosphate buffers in order to cover the pH range studied (6–12) All buffer solutions also contained 0 2 M KCl and 1 mM EDTA

Solutions were made containing approximately 0 05 M DTT^{S}_{S} and 0 25 M GSH, and were made with degeassed buffers from pH 6 to 12 The pH of the solution was adjusted as necessary with degassed 1 M KOH or 1 M HCl by using a Radiometer PHM 84 Research pH meter equipped with an Ingold microelectrode containing an Ag/AgCl reference system Small amounts of DTT^{SH}_{SH} or GSSG were added until their peaks were of comparable height on the HPLC chromatogram to improve the integration accuracy A portion of the reaction mixture was withdrawn and acidified to pH 2 with 6 M HCl prior to HPLC analysis in order to quench the reaction by protonating the thiols Subsequent withdrawals from each reaction mixture were acidified and analyzed until the composition of the mixture remained constant, indicating that equilibrium was achieved At these high concentrations, the equilibria were achieved within 30 min in all cases Results from at least 4 samples were averaged to contribute to each equilibrium constant All studies were carried out at room temperature

A Rainin Dynamax HPLC was used with a Vydac 218TP54 reverse phase C18 analytical column, 0 46×25 cm, for separating the thiol mixtures The flow rate was 1 0 ml min^{-1} of water containing 0 1% (v/v) TFA Absorbance of the eluent was measured by an ISCO V4 absorbance detector monitoring at 220 nm using a 1 mm HPLC flow cell The 4 components in the equilibrium mixtures were nicely resolved as shown in Fig 1 Integrations were carried out using Rainin Dynamax HPLC reprocessing and analysis program

3 RESULTS AND DISCUSSION

The concentrations measured by HPLC peak areas correspond to the total concentrations of each species, irrespective of the extent of ionization of the thiols at that particular pH. This defines the observed equilibrium constant under those particular conditions according to the equation.

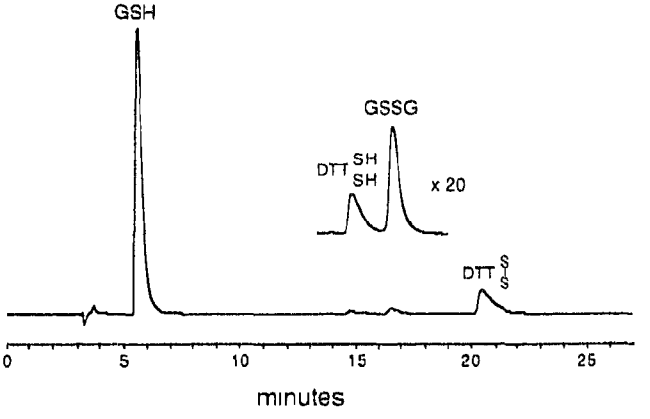

Fig 1 HPLC chromatogram of an equilibrium mixture of GSH, GSSG, DTT^{SH}_{SH}, and DTT^{S}_{S} The sample was kept at pH 7 1 for 1 5 h before acidifying and injecting into the HPLC The concentrations of each species were GSH, 155 mM, GSSG, 1 25 mM, DTT^{S}_{S}, 3 60 mM, and DTT^{S}_{S}, 35 8 mM. The absorbance was measured at 220 nm. The DTT^{S}_{S} and GSSG peaks are also shown expanded 20-fold

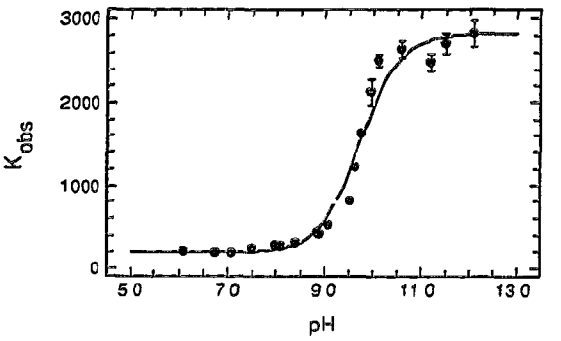

Fig 2 The observed equilibrium constant (K_{obs} in Eqn (2)) measured as a function of pH Error bars not shown are approximately the size of the circles The calculated curve corresponds to values of 200 for K_{SH}, 8 90 for $pK_{a\,GSH}$, 9 15 for $pK_{a\,1}$, and 9 80 for $pK_{a\,2}$ in Eqn (3)

$$K_{obs} = \frac{\{[GSH] + [GS^-]\}^2}{[GSSG]} \times \frac{[DTT^{S}_{S}]}{[DTT^{SH}_{SH}] + [DTT^{S^-}_{SH}] + [DTT^{S^-}_{S^-}]} \quad (2)$$

Values of this observed equilibrium constant as a function of pH between pH 6 and 12 are shown in Fig 2 We estimate the errors in the equilibrium constants to be about 10% considering the accuracy of the calibration of the response factors and measurements made on duplicate samples.

The observed equilibrium constant atany pH, K_{obs}, is related to the equilibrium constant at low pH, K_{SH}, by taking into account the extent to which the thiols are ionized at any particular pH Combining Eqns (1) and (2), we obtain the following equation to express how these two equilibrium constants are related:

$$K_{obs} = K_{SH} \frac{\{1 + K_{a\,GSH}/[H^+]\}^2}{\{1 + K_{a1}/[H^+] + K_{a1}K_{a2}/[H^+]^2\}} \quad (3)$$

where $K_{a\,GSH}$ is the acid dissociation constant for the thiol of GSH, and $K_{a\,1}$ and $K_{a\,2}$ are the macroscopic dissociation constants for the first and second thiols of DTT^{SH}_{SH}, respectively

Several pK_a values have been reported for the thiol of glutathione using a variety of techniques. Some reports found the thiol and amino ionizations to be linked, and thus reported thiol dissociation constants for both a protonated and unprotonated amino group [16,17] Several groups have reported a single pK_a value for the thiol group, independent of ionization of the amino group [8,18–20]. Commonly used values for $pK_{a\,GSH}$ lie in the range of 8.7 to 8.9 [5,8,11] Acid dissociation constants for DTT^{SH}_{SH} have also been reported by several groups, and generally lie in the range of 9.2 to 9.3 for $pK_{a\,1}$ and 10 1 to 10 3 for $pK_{a\,2}$ [21–23].

The effect of pH on K_{obs} can be calculated by using

four parameters: K_{SH}, $pK_{a\,GSH}$, $pK_{a\,1}$, and $pK_{a\,2}$ The data do not allow a unique nonlinear least squares fit of the experimental data [24] to be found. The pK_a values could differ by about 0 3 and K_{SH} could vary by about 10% and still fit the data about equally well A reasonable fit was obtained by using K_{SH}=200, $pK_{a\,GSH}$=8.90, $pK_{a\,1}$=9 15, and $pK_{a\,2}$=9 80 These pK_a values are generally consistent with values reported in the literature. The limiting equilibrium constant at high pH is calculated to be 2800 M These parameters were used to calculate the line drawn in Fig 2 As can be seen in this figure, the calculated curve is slightly broader than the experimental data, possibly because we ignored the effect of the ionization state of the terminal amino group of GSH on $pK_{a\,GSH}$

The observed equilibrium constant between dithiothreitol and glutathione at pH 7 0 from the direct measurement, 200 M, is roughly consistent with that obtained indirectly from our study of the disulfide equilibrium between a protein disulfide and either GSH or DTT^{SH}_{SH}, approximately 120 M at pH 7.0 (unpublished data) We can compare this with previously published values For example, Creighton and Goldenberg [12] indirectly measured the apparent equilibrium constant to be 1160 M at 25°C and pH 8 7 from kinetic studies of disulfide bond formation and breakage during the folding of bovine pancreatic trypsin inhibitor, using both glutathione and dithiothreitol in the kinetic studies From the pH dependence, we calculate a value of approximately 380 M at pH 8 7, about a factor of three lower. This difference corresponds to approximately 0 6 kcal mol^{-1} at 25°C There is more discrepancy between our value of 200 at pH 7 and the value of 8800 M derived by Szajewski and Whitesides [11] using lipoamide–lipoamide dehydrogenase coupled to the formation of NADH. We cannot explain the large discrepancy between this value and our value or that determined by Creighton

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