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Using UV-absorbance of intrinsic dithiothreitol (DTT) during RP-HPLC as a measure of experimental redox potential *in vitro*

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Abstract

Many *in vitro* experiments aimed at studying the response of thiol-containing proteins to changes in environmental redox potentials use dithiothreitol (DTT) to maintain a preset redox environment throughout the experiments. However, the gradual oxidation of DTT during the course of the experiments, as well as the interaction between DTT and other components in the system, can significantly alter the initial redox potential and complicate data interpretation. Having an internal reporter for the actual redox potential of the assayed sample, facilitates the direct correlation between biochemical findings and the experimental redox status. Reversed-Phase High Performance Liquid Chromatography (RP-HPLC) is a widely used, well-established tool for the analysis and purification of biomolecules, including proteins and peptides. Here, we describe a simple, robust, and quantitative RP-HPLC method we developed and tested to determine the experimental redox potential of an *in vitro* sample at the time of the experiment. It exploits the specific UV-absorbance of the oxidized intrinsic DTT in the samples and retains the high resolving power and high sensitivity of RP-HPLC with UV detection.

Keywords

Reversed-Phase High Performance Liquid Chromatography (RP-HPLC); Dithiothreitol (DTT); Redox Potential; Nernst Equation; Disulfide bond; UV-Absorbance

Introduction

Many essential biochemical processes involve proteins that undergo thiol-disulfide interchange reactions. There are recent reviews summarizing the extensively studied role of redox regulation in protein folding [1–4] as well as the rapid advances in redox systems biology that highlight the importance of cellular redox state in maintaining physiologic function [5]. However, the direct measurement of the established redox potentials using electrochemical methods have proven to be difficult due to interactions of the thiols with most electrode surfaces. Consequently, the estimation of standard redox potentials of the sulfhydryl groups of biologically important thiols in the cell have relied on methods involving reversible thiol/disulfide exchange equilibria [6]. Since its first description by Cleland, dithiothreitol (DTT) has become the reagent of choice for reducing disulfides in biochemical research, mainly due to its solubility in water and highly negative redox potential that drives the two step thiol-disulfide exchange reaction to completion [7]. However, DTT is unstable in its reduced form over extended periods of time, and its

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oxidation can be further catalyzed by metal ions [8]. Since it is difficult to conduct *in vitro* experiments under predetermined redox potentials using DTT, the total initial DTT concentration $[DTT_0]$ is typically reported and the experiment is assumed to maintain a constant redox potential under anaerobic conditions in the presence of a chelating agent such as EDTA. However, for experiments that require various metal ion and/or protein thiol concentrations monitored over an extended period, the developed redox potential can change significantly from the predetermined value. Hence, it is of great scientific interest to have a simple, sensitive, and quantitative method that relies on intrinsic data from the assayed sample to monitor experimental redox potential.

RP-HPLC is a widely used, well-established tool for the analysis and purification of biomolecules, including proteins and peptides. It has an extremely wide application base in protein biochemistry due to its ability to separate polypeptides of nearly identical sequences and its sensitivity to protein conformation. Recently, it has regained attention for its coupled use with mass spectroscopy in the separation and analysis of peptide fragments from enzymatic digests including of protein therapeutic products yielding to hundreds of patents and applications per year within the last decade [9]. Today most polypeptide separations are performed on large pore size (200 Å) columns with a linear aliphatic hydrocarbon group forming the hydrophobic phase (C4, C8, or C18). C18 columns are generally preferred for peptides and small proteins or proteins protein domains less than about 5,000 daltons, as typical for the above mentioned applications. Here, we describe an RP-HPLC based methodology using the UV-absorbance data at 280 nm from the oxidized DTT (DTT_{ox}) within an assayed protein sample analyzed on an analytical Agilent ZORBAX large pore C18 column to determine experimental redox potential. We demonstrate that, in addition to being both sensitive and quantitative, this simple method can be directly incorporated into a fully automated data collection, processing, and analysis scheme since it does not require any additional sample preparation or monitoring.

Oxidation-reduction reactions involving proteins with vicinal thiols that form disulfide bonds are two electron transfer reactions. The redox potential (E_h) of such a system is calculated according to the Nernst equation: $E_h = E_0 + (RT/nF) \ln ([DTT_{ox}]/[DTT_{red}]^2)$ [10]. In this expression, E_0 is the standard potential for the redox couple at the defined pH, $R = 8.314 \text{ J/mol.K}^\circ$, T is the absolute temperature, $F = 95484.6 \text{ J/V.mol}$, and n is the number of electrons transferred. For the *in vitro* system described below involving the thiol exchange between DTT and protein at 20 C° , the Nernst equation simplifies to:

$$E_h = E_0 + 29.4 \log ([DTT_{ox}]/[DTT_{red}]^2) \quad (1)$$

where $[DTT_{red}]$ and $[DTT_{ox}]$ are molar concentrations and E_0 is calculated to be -354 mV at experimental pH 7.7, using -332 mV for pH 7.0 and -366 mV for pH 8.1 [7], with an adjustment of $-3.4 \text{ mV/0.1 pH unit}$. Using this equation, the experimental redox potential can be calculated, provided that both $[DTT_{red}]$ and $[DTT_{ox}]$ can be accurately determined.

Even the ultra-high grade DTT_{red} (Sigma, cat#: 43815) typically used in reduction experiments, can contain up to 0.5% DTT_{ox} and becomes further oxidized over time, even when stored at -20 C° under dry conditions. Hence, the actual DTT concentration of a DTT_{red} solution prepared from DTT_{red} solid is described by $DTT_{total} ([DTT_{red}] + [DTT_{ox}])$. In contrast, the calculated $[DTT_{ox}]$ of an oxidized DTT solution prepared from DTT_{ox} solid is the true $[DTT_{ox}]$.

The reverse-phase HPLC (RP-HPLC) based procedure described here was developed for studies involving the reduction of a homologous set of small, protein modules called Lin12/Notch Repeats that contain three sets of unique disulfide bonds and coordinate a Ca^{2+} ion

[11]. The goal was to find a direct and sensitive reporter of the actual redox potential at different time points throughout the experiment to verify that the experiment was carried out under a constant redox potential or to quantify the magnitude of any change from the starting conditions. The developed method was then tested for three separate applications where the experimental variables were chosen to represent some of the most common biochemical studies involving *in vitro* protein reduction. Since the methodology is solely based on the UV-absorbance of the oxidized DTT in the sample and no other sample component, it can easily be extended to a wide range of chromatographic assays involving a DTT-containing sample monitored via UV absorbance between 260 and 320 nms.

Experimental

Reagents and Samples

HPLC mobile phases used HPLC-grade acetonitrile (EMD Chemicals), water purified by Milli-Q water system (Millipore, USA) and trifluoroacetic acid (TFA) (Acros Organics). A 10 mM stock standard solution of DTT_{ox} was prepared by dissolving DTT_{ox} solid (EMD, cat #: EM 3860) in 20 mM sodium hydroxide and then sequentially diluted 1:10 with mobile phase A (5% Acetonitrile, 95% H₂O, 0.1% TFA) to obtain standard solutions of 1 mM, 100 μ M, and 10 μ M DTT_{ox}. All other reagents used in the test experiments were ACS Grade and >99% unless indicated otherwise: HEPES (Acros Organics), sodium chloride (MP Biomedicals), and -DL-dithiothreitol (Biotechnology grade, Amresco), ethylenediaminetetraaceticacid (EDTA) (Acros Organics), calcium chloride dihydrate (BDH), nickel (II) sulfate hexahydrate, (Aldrich), cobalt chloride (Mallinckrodt), magnesium chloride anhydrous (Sigma), zinc chloride (Fisher Chemicals), terbium (III) chloride hydrate (Aldrich)

Recombinant human Notch1 LNRA (hN1LNRA) used in this work was expressed in *E.coli* and purified as described in detail in the Electronic Supplementary Material. For the reduction experiments, the lyophilized oxidized hN1LNRA was resuspended in H₂O to a stock concentration of ~ 150 μ M and dialyzed overnight against 20 mM HEPES pH 7.7, 150 mM NaCl and the chosen metal (5 mM CaCl₂, NiSO₄, CoCl₂, TbCl₃, MgCl₂) or chelator (1 mM EDTA)). Protein concentration was determined by UV absorbance at 280 nm using an extinction coefficient $\epsilon = 5875 \text{ M}^{-1}\text{cm}^{-1}$.

Protein Reduction Experiments

Prior to each reduction experiment, the dialyzed stock protein solution was diluted to a final concentration of ~15 μ M using the corresponding dialysis buffer. For all the experiments performed under anaerobic conditions, the dialyzed protein sample, as well as the reduced DTT stock was flushed with N₂ and placed in an Atmos Bag (Sigma Aldrich, Cat #: Z530212) that was purged continuously with N₂ for 15 minutes prior to the start of the experiment. Reduction was initiated with the addition of 35 μ l of the reduced DTT stock to each 140 μ l aliquots of 15 μ M protein sample to yield a final DTT concentration of 2.5 mM. The thiol-exchange was quenched at indicated time points by acidifying the samples with 2.75% trifluoroacetic acid (TFA) (for a final concentration of 1% TFA) acid. The reduction experiments under aerobic conditions were set up on the bench using identical sample compositions without the N₂ purging.

Data collection and Processing

Quenched samples were assayed by Reverse-Phase High Performance Liquid Chromatography (RP-HPLC) using a Waters 600E Pump/controller system equipped with an analytical C18 column (Agilent ZORBAX StableBond SB-300 Å; 5 μ m particle size, 300 Å pore size, 4.6 $\times$ 250 mm). After filtering through UltraFree-MC centrifugal units

(Millipore, Cat#UF30GVNB) 200 μ l of ~ 10 μ M protein was loaded directly onto the column equilibrated in Mobile phase A (5% Acetonitrile, 95% H₂O, 0.1% TFA) using a Waters 717Plus Autosampler, and was eluted from the column during a 15 minute slow linear gradient from 19%–22% Mobile Phase B (5% H₂O, 95% Acetonitrile, 0.1% TFA). The HPLC chromatograms at 280 nm were recorded on a Waters 2998 Photodiode Array (PDA) detector and were processed using Waters Empower software with apex peak detection option and peak integration with optimized parameters to ensure non-overlapping peak area selections.

Data Analysis

To convert integrated areas (IA₂₈₀) for DTT_{ox}, to concentrations in subsequent reduction experiments, the relationship $IA_{280} = CF_{280} \times [DTT_{ox}]$ was used. The slope of the linear fit line to the plotted standard curve of integrated area (IA₂₈₀) vs. predetermined standards of [DTT_{ox}] was assigned the conversion factor, CF₂₈₀. Experimental [DTT_{red}] was calculated by subtracting [DTT_{ox}] from [DTT₀]. The redox potentials for each experiment at different time points were calculated using equation (1).

To compare redox potentials between two single experiments, the percent difference was calculated using:

$$\left| \frac{x_1 - x_2}{x_{average}} \right| \times 100\% \quad (2)$$

where x_i and $x_{average}$ are the calculated redox for any given experiment and the average potential of the compared experiments, respectively.

To determine if there was any significant difference between two sets of triplicate experiments, an unpaired t-test was performed at a confidence level of 95% ($t_{table} = 2.776$) using:

$$t_{calc} = \frac{|\bar{x}_1 - \bar{x}_2| * \sqrt{\frac{(N_1 * N_2)}{(N_1 + N_2)}}}{s_p} \quad (3)$$

where $t_{calc} < t_{table}$ statistically not different and $t_{calc} > t_{table}$ statistically different.

Results and Discussion

Method development

Due to its ring structure, DTT_{ox}, strongly absorbs in the UV region, with a maximum around 283 nm, while DTT_{red}, shows a significant absorbance only below 260 nm (Figure 1, inset 1) [7]. By limiting the UV-detection window to the 260–320 nm range during data acquisition, an HPLC chromatogram that selects only for the DTT_{ox} signature peak can be obtained. This DTT_{ox} peak is integrated to quantify the amount of oxidized DTT species present in the sample. A set of calibration experiments performed using predetermined [DTT_{ox}], in the absence of protein, are used to plot a standard curve that relates integrated areas (IA₂₈₀) to the corresponding [DTT_{ox}]. For the applications described in this work, the standard curve of integrated area (IA₂₈₀) vs. [DTT_{ox}] was plotted for the [DTT_{ox}] range of 10 μ M to 10 mM DTT_{ox} in 10 fold increments (Figure 1). The slope of the linear fit to the standard curve was assigned as the conversion factor (CF₂₈₀) between IA₂₈₀ for DTT_{ox} and [DTT_{ox}] for all subsequent calculations ($IA_{280} = CF_{280} \times [DTT_{ox}]$) from experimental data. Subsequent blank runs after each DTT_{ox} run confirmed that there was no retained detectable DTT_{ox} stuck to the column. Since [DTT_{tot}] in the assayed sample is equal to the initial total

DTT concentration, $[DTT_0]$, the $[DTT_{red}]$ was calculated by subtracting $[DTT_{ox}]$ from $[DTT_0]$. Finally, these experimentally determined concentrations were substituted in equation (1) to calculate the experimental redox potentials.

For automated peak identification and integration, it was critical that the mobile and stationary phases of the chromatographic system, as well as the elution method, yielded well-resolved, non-overlapping protein and DTT_{ox} peaks. We tested a variety of mobile phases and elution methods and determined that DTT_{ox} always eluted around 10.0 minutes in our system indicating that the retention of the DTT species was independent of the chosen mobile phase or the specific elution method (see Electronic Supplementary Material, Figure S1). This finding ensures that an optimized elution method where the DTT_{ox} always eluted before the proteins and did not overlap with any other sample can be developed extending the applicability of the described methodology. For the reduction experiments that motivated this study, the final elution method was chosen based on its efficacy in separating the reduced hN1LNRA from its oxidized form in the sample soon after the elution of the DTT_{ox} peak used as the redox reporter. It consisted of an initial 3 minute wash in Mobile Phase A and a 2 minute steep linear gradient from 0%–19% Mobile Phase B followed by a 15 minute slow linear gradient from 19%–22% Mobile Phase B at a flow rate of 1 ml/min.

Tested Applications

Figure 2 is a representative set of HPLC chromatograms from the reduction of hN1LNRA saturated with 5 mM Ca^{2+} , under anaerobic conditions, and quenched at 0, 15 min, 30 min, 60 min, and 180 min after the addition of 2.5 mM DTT. Results show a gradual decrease in the amount of oxidized hN1LNRA with a corresponding increase in its reduced form, accompanied by a quantifiable increase in $[DTT_{ox}]$. To determine if the observed increase in $[DTT_{ox}]$ resulted in a significant change in the established redox potential across equivalent experiments quenched at different time points, we first calculated the redox potential as described above (Table 1, panel A). We then used these calculated values to determine the percent difference between pairs of experiments using average redox potentials from triplicate experiments and to perform an unpaired t-test on triplicate experiments (Table 1, panel B). While small sample sizes used for the calculations precluded a thorough statistical analysis, the results clearly showed a significant change in the established redox potential within the first 15 minutes of the experiment despite anaerobic conditions. Even though the redox potential continued to increase steadily throughout the duration of the experiment (3 hours), the increase was much more gradual after the initial increase, resulting in a significant change only over a 2.5 hour or longer time period.

When the same experiments were repeated under aerobic (bench-top) conditions, the calculated percent difference in the established redox potentials demonstrated that despite the big difference in the established starting redox potentials between the aerobic and anaerobic conditions, the rate of increase was similar over the course of the experiment (Table 1, panel C). This finding brings into question the requirement for conducting similar experiments under anaerobic conditions. At least for relatively short experiments, it might be possible to eliminate the need of special equipment to ensure a steady redox status as long as the initial difference is compensated for, by perhaps starting with a higher $[DTT_0]$ and actual redox potential is monitored and recorded as a part of data collection.

Many *in vitro* biochemical assays include metal ions due to their structural or functional roles for the protein under investigation. It has been shown that DTT is a very efficient chelating agent for a wide range of biologically relevant metal ions [12]. While certain organic compounds such as quinones are known to oxidize DTT very effectively, the extent of DTT oxidation due to the presence of transition metals and chelating agents is less well established. A recent study investigating the impact of soluble transition metals in fine

particulate matter on its oxidative potential, which has been linked to the adverse health effects, showed that many of the tested transition metals did oxidize DTT to varying degrees [13]. Therefore, we also tested our method in a series of reduction experiments that contained various metal ions commonly used in biochemical studies, as well as Ethylenediaminetetraacetic acid (EDTA) under anaerobic conditions. Our results clearly show that certain metals such as Ni^{2+} and Co^{2+} oxidize DTT to a much higher degree than others, such as Ca^{2+} , Mg^{2+} , and Tb^{2+} , and that EDTA can further decrease the amount of oxidized DTT in the blank buffer, likely because it chelates trace levels of metals that may exist in the buffer (Table 1, panel D). We believe that the significance of this effect is extremely context dependent, i.e., a 3–5% increase in the actual redox potential seen for Ni^{2+} and Co^{2+} in our test experiment might be negligible for certain experimental designs, but might be very significant for others. Hence, the ability to quantitate this impact is highly valuable.

Conclusions

Due to its highly negative redox potential, DTT is the preferred reducing agent in many *in vitro* redox experiments. The goal of this study was to develop a chromatographic method to accurately quantify the changes in the *in vitro* experimental redox status set by an initial predetermined $[\text{DTT}_0]$ during the course of a protein reduction experiment under a variety of conditions. The results presented here underscore the importance of having a simple, sensitive, and quantitative method to monitor the experimental redox potential during *in vitro* experiments presumed to maintain a constant redox potential set by the $[\text{DTT}_0]$. The described methodology utilizes data from the DTT_{ox} component of the chromatographic separation assay monitored via UV-based detection in the 260–320 nm range and provides a unique opportunity to assess the importance and/or significance of variations in experimental conditions on redox potential. The method is highly sensitive, with 20 pg of DTT_{ox} yielding reliable and reproducible peak integrations at 280 nm. Finally, the procedure can be easily incorporated into an automated data processing scheme and facilitate direct comparison of results across different experiments, enhancing the reliability of data interpretation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. The standard curve of integrated area (IA_{280}) vs $[DTT_{ox}]$

A conversion factor (CF_{280}) of 3.67×10^9 between the IA_{280} and $[DTT_{ox}]$ was determined from the linear fit to the curve. Both axes are plotted with a logarithmic scale. Inset 1, (adapted from [7]): The absorbance of DTT_{ox} (—) in the ultraviolet region with an absorbance maximum at 283 nm and DTT_{red} (----) with negligible absorbance above 260 nm. Inset 2: Overlay of the 280 nm HPLC chromatograms corresponding to the integrated DTT_{ox} peaks for 10 mM, 1 mM, 100 μ M, and 10 μ M DTT_{ox} .

Figure 2. Representative set of reduction experiments

Overlay of one representative set of 280 nm HPLC chromatograms for the reduction of 15 μ M hN1LNRA in 20 mM HEPES (pH 7.7), 150 mM NaCl, 5 mM CaCl₂, and 2.5 mM initial DTT acidified with 1% TFA at the indicated time points. The gradual increase in the [DTT_{ox}] is highlighted in the inset and is accompanied by a decrease in the oxidized hN1LNRA and an increase in its reduced form. * indicates a non-thiol containing species, not impacted by DTT. IA₂₈₀ for DTT_{ox} from these chromatograms are used to calculate redox potentials in Table 1.

Table 1
Impact of different experimental variables on the redox status of the sample

Panel A: Summary of calculated [DTT_{ox}] and redox potentials for a triplicate set of test experiments represented in Figure 2 and described in the text. Panel B: The pair-wise comparison of the calculated redox potentials at different time points and whether the change between two sets of triplicate experiments was significant (*) or not (-) based on the unpaired t-test performed at a 95% confidence level. Panel C: Percent increase in calculated redox potentials at different time points for identical set of experiments conducted under aerobic vs. anaerobic conditions. Panel D: Summary of calculated [DTT_{ox}] and redox potentials for a duplicate set of test experiments performed under anaerobic conditions in the absence of any protein or chelator/metal (buffer), and in the presence of protein and one of the following chelator./ metal: Ca²⁺, Tb³⁺, Mg²⁺, Ni²⁺, Co²⁺

A.		
Calculated redox potentials for triplicate samples containing [DTT ₀] = 2.5 mM, 15 μ M hN1LNRA, 20 mM HEPES pH 7.7, 150 mM NaCl, 5 mM CaCl ₂		
Time (min)	[DTT _{ox}] (μ M)	Redox Potential (mV)
0	3.8 $\pm$ 1.9	-360.9 $\pm$ 5.9
15	11.4 $\pm$ 4.2	-347.3 $\pm$ 4.7
30	14.2 $\pm$ 5.5	-344.7 $\pm$ 4.8
60	20.3 $\pm$ 9.1	-340.6 $\pm$ 5.5
180	39.0 $\pm$ 14.4	-332.3 $\pm$ 4.6

B.		
Pairwise comparison of the calculated redox potentials under anaerobic conditions		
Time points (min)	% increase	Statistical Significance
0 & 15	3.8	*
0 & 30	4.6	*
0 & 60	5.8	*
0 & 180	8.3	*
15 & 30	0.8	-
15 & 60	1.9	-
15 & 180	4.4	*
30 & 60	1.2	-
30 & 180	3.7	*
60 & 180	2.5	-

C.	
Comparison of the calculated redox potentials for experiments carried out under anaerobic vs aerobic conditions	
Time (min)	% Increase
0	17.48
15	14.39
30	14.12
60	13.40

C.	
Comparison of the calculated redox potentials for experiments carried out under anaerobic vs aerobic conditions	
Time (min)	% Increase
180	11.55

D.		
Calculated redox potentials for duplicate samples containing [DTT₀]= 2.5 mM, 15 μM hN1LNRA, 20 mM HEPES pH 7.7, 150 mM NaCl, 5 mM various metals after min of reduction		
Metal	[DTT_{ox}] (μM)	Redox Potential (mV)
Buffer	199.9 $\pm$ 25.1	-478.2 $\pm$ 1.8
EDTA	134.1 $\pm$ 12.1	-483.6 $\pm$ 1.2
Ca ²⁺	215.3 $\pm$ 4.2	-477.1 $\pm$ 0.3
Tb ³⁺	243.2 $\pm$ 3.8	-475.4 $\pm$ 0.2
Mg ²⁺	257.4 $\pm$ 47.7	-474.6 $\pm$ 2.7
Ni ²⁺	534.0 $\pm$ 40.9	-462.8 $\pm$ 1.4
Co ²⁺	585.8 $\pm$ 80.0	-461.1 $\pm$ 2.6