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A chromogenic assay of substrate depletion by thiol dioxygenases

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ABSTRACT

A fast and easy method for enzyme activity assays using the chromogenic Ellman reagent, 5,5′-dithiobis(2-nitrobenzoic acid), was developed. The method was used to measure the activity of the nonheme mono-iron enzyme cysteine dioxygenase. Quantifying the depletion of the substrate, cysteine, allowed standard kinetic parameters to be determined for the enzyme from *Rattus norvegicus*. The assay was also used to quickly test the effects of ionic strength, pH, enzyme storage conditions, and potential inhibitors and activators. This assay facilitates a higher throughput than available HPLC-based assays, as it enjoys the advantages of fewer sample handling steps, implementation in a 96-well format, and speed. In addition, the relative specificity of Ellman's reagent, coupled with its reaction with a wide range of thiols, means that this assay is applicable to many enzymes. Finally, the use of readily available reagents and instrumentation means that this assay can be used by practically any research group to compare results with those of other groups.

Thiol dioxygenation is an irreversible and physiologically important step in sulfur metabolism. Three enzymes have been found capable of adding molecular oxygen to a sulfhydryl group to form a sulfinic acid: cysteine dioxygenase (CDO,[1] EC 1.13.11.20) [1], cysteamine dioxygenase [2], and 3-mercaptopropionic dioxygenase [3]. Each is reported to be highly specific for a single substrate. Active research into the mechanism and structural basis of catalysis by these enzymes depends on availability of comparable kinetic data for a range of assay conditions and a variety of enzymes from different species and mutagenic procedures.

Multiple methods for studying the dioxygenation reaction are reported in the literature. For cysteine dioxygenation, the most common methods monitor the levels of the reaction product, cysteine sulfinic acid (CSA), after separation by HPLC. However, sample preparation, chromatographic separation, and detection all differ considerably. The reaction is quenched variously with acid [1,4–6], base [7], flash freezing [8], or acetone [9,10] and in some protocols is followed by derivatization for fluorescence detection [1,4,7]. Detection of underivatized product and substrate is possible by absorbance at 215 nm [5,6,8] or with an evaporative light-scattering detector (ELSD) [9,10]. As the detection methods used in these assays do not discriminate between product and substrate, chromatographic separation is required; reverse-phase and hydrophilic–interaction liquid chromatography have been used. The only reported cysteamine dioxygenation assay acid quenches the reaction, derivatizes the product, and identifies it through HPLC analysis [2]. HPLC (coupled to a refractive index detector) was also used for measurement of 3-MPA dioxygenation after quenching by heat [3]. HPLC separation reduces the background signal, so it can give enhanced sensitivity as well as identification of products by retention time. However, these methods are complicated by sample preparation requirements to derivatize the product and to remove assay components (such as buffer salts or enzyme) before chromatography. All those factors can compromise quantitation and therefore limit the ability to compare results between different techniques and different research groups, especially when a particular detector is required. In addition, HPLC methods are generally optimized for one substrate or its reaction product and may not be easily applied to the study of alternate substrates and products. NMR was also utilized to observe dioxygenation [3,9] but is more suited to initial identification of reaction products, rather than routine kinetic measurements, as sample preparation and measurements can be highly demanding. Oxygen electrodes can be used for continuous detection of the consumption of the second substrate of the reaction, molecular oxygen [7,11], but this approach can be limited either by the solubility of oxygen in aqueous

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[1] *Abbreviations used:* 3-MPA, 3-mercaptopropionic acid; BCS, bathocuproine disulfonic acid; CDO, cysteine dioxygenase; CSA, cysteine sulfinic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTPA, diethylenetriaminepentaacetic acid; ELSD, evaporative light-scattering detector; ferrozine, 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulfonic acid sodium salt.

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solutions or the need to control the reintroduction of oxygen by diffusion. In addition, controlling for nonspecific oxygen consumption can be challenging. Another method for qualitative analysis quenches the reaction by heat followed by thin-layer chromatography but it has not been developed for quantitation of dioxygenation [12]. None of the established methods scales to satisfy a high-throughput of samples and none transfers easily from laboratory to laboratory to allow comparison.

The free sulfhydryl group of thiols can be quantified by the widely used Ellman's reagent, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) [13]. The substantial enhancement of absorbance at 412 nm upon reaction of DTNB with a thiol gives simple and selective quantification of practically any thiol and is therefore suited to be applied to a fast and facile assay of thiol dioxygenation as well. This reagent has been used to study other enzyme activities, including peptide deformylase [14], glucosamine-6-phosphate synthase [15], cystine lyase [16], or horseradish peroxidase [17]. For use with thiol dioxygenases, the derivatization of the substrate thiol for quantitation simultaneously quenches the reaction. High reactivity and a large extinction coefficient provide good sensitivity.

Here we report a facile 96-well format Ellman assay of *Rattus norvegicus* CDO activity. Comparison to the HPLC-based method also used in our lab [9] confirmed that cysteine depletion measured with this method corresponds to cysteine depletion and CSA production observed with an ELSD detector. We have exploited the broad reactivity of DTNB to assess CDO specificity for cysteine compared to 3-MPA, a determination that has been addressed only partially in the literature [18]. In addition we use this assay as an easy screen of the effects of variations in reaction conditions and enzyme storage conditions as well as the activation or inhibition effect of certain chemicals on the enzymatic activity. This facile method should be a useful innovation as it can easily be applied to many thiol dioxygenases using equipment found in almost all biochemical laboratories.

Materials and methods

General materials

3-MPA, L-cysteine, ferrozine, DTNB, and diethylenetriaminepentaacetic acid (DTPA) were purchased from Sigma–Aldrich (USA). EDTA was purchased from Ajax Finechem (Australia). Ascorbic acid was purchased from Thermo Fisher Scientific (Europe). All other chemicals used are listed in our previous publication [9].

Overexpression and purification of CDO

The *R. norvegicus* CDO was expressed and purified using Strep-Tag affinity technology as previously reported by our group [11]. The purified protein preparations were dialyzed extensively (>10^9 dilution factor) against buffer containing 10 mM phosphate (pH 7.5) and 20 mM NaCl. After concentration, protein preparations were at least 95% pure and contained ~15% mol/mol endogenously bound iron. The freshly purified protein was reconstituted with iron to >70% mol/mol with ammonium iron(II) sulfate followed by treatment with Chelex [11] to remove unbound excess iron. The iron concentrations were determined by a colorimetric assay using ferrozine [11]; enzyme concentrations in all kinetic calculations refer to the concentration of iron bound in the active site, rather than the enzyme polypeptide concentration.

Discontinuous enzyme assay using Ellman's reagent

Ellman's reagent was prepared with 0.64 mM DTNB in aqueous solution containing 100 mM phosphate buffer (pH 7.5) and stored for no longer than 2 weeks at 4 °C. Ellman's reagent (97.5 μl) was pipetted into the wells of a 96-well flat-bottom polystyrene plate (Corning) before the start of each assay.

Standard assay reactions contained 5 mM L-cysteine, 100 mM phosphate, pH 7.5, 20 mM NaCl, 0.1 mM bathocuproine disulfonic acid (BCS), 7 μM CDO at 37 °C, similar to our previous work [9,10]. Standard reactions were performed in a volume of 250 μl under constant stirring in open 1.5-ml tubes submerged in a water bath and 12 time-point samples of 2.5 μl were withdrawn at intervals of 120 s. These standard assay conditions were varied to test the effects of specific conditions and all variations are noted in the text and figure legends as appropriate. All stock solutions were prewarmed to the assay temperature. Stock solutions of L-cysteine or 3-MPA were always prepared fresh and further diluted within minutes. First the thiols were dissolved in the appropriate buffer and adjusted to the desired pH. The stock was then diluted with buffer of the same concentration and pH (and containing inhibitor when indicated) to the desired concentrations. The reaction was initiated by adding CDO to the cysteine or 3-MPA solution. At specific time points 2.5-μl aliquots were withdrawn from the reaction mixture and placed into a well of a 96-well plate containing Ellman's reagent to generate a final dilution factor of 40. This large dilution factor and the high buffer concentration (100 mM) ensured that the final pH remained 7.5 so that accurate thiol concentrations could be determined. The absorption at 412 nm was measured on a Thermo Scientific Multiskan GO UV/Vis microplate spectrophotometer. The absorption at 412 nm stayed constant for at least 24 h, suggesting that the quenching of the enzymatic reaction was practically immediate and that the products remain stable, so long as exposure to direct sunlight was avoided. All readings were corrected for the absorbance of Ellman's reagent in buffer alone. Absorption at 750 nm was monitored to control for artifactual light scattering (e.g., from bubbles). Although CDO contains cysteine residues, for the concentrations used in the assay the resulting absorption at 412 nm was negligible. Ellman's reagent incubated with CSA, cystine, EDTA, ascorbic acid, DTPA, glycerol, and BCS showed no enhanced absorption at 412 nm.

For each rate determination, 12 time points were collected, at intervals of 120 s, and a linear regression was used to determine the rate of thiol depletion and to verify the linearity of the reaction over the selected time course. A standard curve for cysteine or 3-MPA with Ellman's reagent was prepared in parallel with each experiment. The reproducibility of the standard curve confirmed the calculated concentrations of cysteine and 3-MPA. In addition, reactions lacking CDO but otherwise subjected to the same process showed no decrease in the corresponding thiol over ~2000 s, suggesting no formation of cystine. The kinetic data were analyzed by Prism 5 software (GraphPad Software). Error bars and numerical errors represent the standard error of the mean, the range, or the error associated with the fit, as indicated.

HPLC-based assay

In parallel with the cysteine depletion measured by the Ellman assay, enzymatic activity was confirmed quantitatively using HPLC-ELSD as previously described [9]. As larger volumes per time point are required here, only seven time points in 180-s intervals were collected.

Results and discussion

Comparison of the Ellman assay with HPLC-ELSD

The Ellman assay revealed a constant rate of substrate depletion over many minutes; an exemplary time course is shown in Fig.1.