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A highly sensitive colorimetric microplate ferrocyanide assay applied to ascorbate-stimulated transplasma membrane ferricyanide reduction and mitochondrial succinate oxidation

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Abstract

Ferricyanide reduction frequently is analyzed to determine the activity of membraneous reductases. An improved, highly sensitive, and rapid method for quantitative endpoint determination of ferrocyanide is presented. Ferrocyanide is oxidized by Fe^{3+} in the presence of Ferene-S under acid conditions to form a chromogenic Ferene-S/Fe^{2+} complex. The latter is quantitated at 593 nm with a sensitivity of 33.2 $mM^{-1} \cdot cm^{-1}$. The assay is 60% more sensitive to ferrocyanide (and with a 50% lower detection limit) than the prevailing method of Avron and Shavit, which employs sulfonated bathophenanthroline as the ferrous chromogen. Both pH dependence and potential sources of interference are discussed. Using the method, a sulfhydryl-sensitive, ascorbate-stimulated transplasma membrane ferricyanide reductase was assayed in human chronic myeloid (K562) leukemia cells. Furthermore, malonate-sensitive succinate dehydrogenase activity of heart mitochondria was easily assayed with ferricyanide as terminal electron acceptor. The current method will suit routine applications demanding high throughput, robustness, and sensitivity in a 96-well plate format.

Keywords: Ferricyanide; Ferene-S; Transplasma membrane electron transport; Ascorbate; Dehydroascorbic acid; Succinate dehydrogenase

Enzyme-catalyzed oxidoreduction reactions are ubiquitous throughout life, playing crucial roles in nearly all cellular processes. The trivalent ferricyanide (FIC)[1] anion is commonly used as an artificial electron acceptor for the assay of biological oxidation–reduction reactions due to its high redox potential ($E_0' = 360$ mV), excellent aqueous solubility, and lipid bilayer impermeance. Reduction of FIC to ferrocyanide (FOC) can be quantitated by the loss of absorbance at 420 nm ($\varepsilon_{420\ nm} = 1\ mM^{-1} \cdot cm^{-1}$) [1] but suffers from poor sensitivity due to the low extinction of FIC at this wavelength.

In 1963, Avron and Shavit presented a simple and sensitive method for endpoint quantitation of FOC [2]. Their method, which is applicable to FOC concentrations greater than 1 μM, depends on the reduction of ferric iron to ferrous iron by FOC [3], followed by chelation of ferrous iron by bathophenanthroline disulfonic acid (BPS) and quantitation at 535 nm. The resultant sensitivity to FOC is 20.5 $mM^{-1} \cdot cm^{-1}$ [2]. This method has been widely used as an alternative to direct spectrophotometric quantitation of FIC. Here we present a novel strategy for FOC quantitation in a microplate-based format that replaces BPS with Ferene-S (FS) under more acidic conditions, increasing sensitivity to 33.2 $mM^{-1} \cdot cm^{-1}$ and decreasing the lower detection limit to 0.5 μM FOC.

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[1] *Abbreviations used:* FIC, ferricyanide; FOC, ferrocyanide; BPS, bathophenanthroline disulfonic acid; FS, Ferene-S; tPMET, transplasma membrane electron transport; AcOH, acetic acid; TCA, trichloroacetic acid; PBS, phosphate-buffered saline; ANOVA, analysis of variance; FZ, ferrozine; OP, *o*-phenanthroline; NTA, nitrilotriacetate; LLoD, lower limit of detection; EDTA, ethylenediamine tetraacetic acid; EGTA, ethylene glycol tetraacetic acid; β-ME, 2-mercaptoethanol; DTT, dithiothreitol; SVCT, sodium-ascorbate cotransporter; DHA, dehydroascorbic acid; AFR, ascorbate free radical; *p*CMBS, *p*-chloromercuribenzene sulfonate; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; SDH, succinate dehydrogenase.

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Transplasma membrane electron transport (tPMET) is ubiquitous in eukaryotes, representing a conserved mechanism for plasma membrane-mediated redox regulation of cellular metabolism [4,5]. Intriguingly, tPMET is recognized as playing important roles in various disease states, including neurodegeneration [6] and cancer progression [7]. Although first described more than 30 years ago, the molecular events involved in tPMET remain largely uncharacterized [5]. One reason for this is the absence of a simple yet sufficiently sensitive method for analysis of enzyme activity. Interestingly, human erythrocytes possess a tPMET system that uses cytosolic ascorbate as the major electron donor to reduce extracellular FIC [8,9]. Whether ascorbate is a significant electron donor for tPMET activity for other cell types is unclear. Using the current method, a greatly enhanced rate of transplasma membrane electron transport to extracellular FIC was demonstrated for human chronic myeloid leukemia (K562) cells after preloading with ascorbate, supporting the notion that intracellular ascorbate may be a physiologically significant reductant for tPMET in nucleated cells. This method will be useful for analyzing the existence of similar systems in cells from other lineages and/or disease models.

This article presents a significantly improved method for determination of micromolar concentrations of FOC following FIC reduction. Advantages of the described method include its simplicity, sensitivity, and suitability to high-throughput applications.

Materials and methods

Unless otherwise stated, all chemicals were obtained from Sigma–Aldrich and Fluka (Castle Hill, Australia). Glacial acetic acid (AcOH, ~17.5 M) and ferric chloride were obtained from Merck (BDH AnalaR, Kilsyth, Australia). Microplate assays were performed on a Benchmark Plus microplate spectrophotometer (Bio-Rad, Regents Park Industrial Estate, Australia) using Nunc 96-well flat-bottom transparent plates with a final assay volume of 0.25 ml (path length = 0.75 cm). Where indicated, spectrophotometric readings were also performed with an Aminco DW2 Olis conversion dual-beam UV/Vis spectrophotometer. All solutions were constructed with deionized water.

Determination of FOC: The general procedure

The following working solutions were constructed and stored at 4 °C for a maximum of 1 month: (A) 3 M sodium acetate, adjusted to pH 6.0 at room temperature with AcOH; (B) 0.2 M citric acid; (C) 3.3 mM ferric chloride in 0.1 M AcOH (constructed periodically by a 1:9 dilution of 33 mM ferric chloride in 1 M AcOH), protected from light; and (D) 20 mM FS, protected from light. To determine FOC levels in a typical experiment, 125 μl of sample containing up to 150 μM FOC were added to wells in a 96-well plate and then acidified with 25 μl of AcOH and/or trichloroacetic acid (TCA) ($[AcOH]_f = 7.7\%$ and/or $[TCA]_f \leqslant 3.8\%$). FS developer solution was constructed immediately before use by combining solutions A to D in the ratio 2:2:2:1 (A:B:C:D). 100 μl of developer were then added to each sample (final volume = 250 μl). Plates were agitated at 600 rpm at room temperature for 40 min while protected from light. Absorbance readings were then taken at 593 nm in a microplate spectrophotometer. Where indicated, slight variations from this general procedure were employed. Using the sensitivity of 33.2 $mM^{-1} \cdot cm^{-1}$ determined here (Table 1) for the FS/Fe^{2+} complex at 593 nm, the micromolar FOC concentration was obtained by multiplying A_{593} for 250 μl of solution (path length = 0.75 cm) by a factor of 80.3. Variations in this procedure resulting in changes in relative volumes were reflected by a change in this factor. Readings were always zeroed against an equivalent volume of buffer containing no FOC but were treated exactly the same as for the FOC-containing samples.

K562 cells and culture conditions

The human chronic myeloid leukemia cell line, K562 (provided by Des Richardson, University of Sydney), was maintained in exponential growth in RPMI 1640 (Gibco, Australia) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gibco), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 100 μg/ml erythromycin in a humidified incubator at 37 °C and 5% ambient CO_2. Prior to an assay, cells were harvested and washed twice in cold phosphate-buffered saline (PBS, pH 7.4) with or without 10 mM D-glucose (Merck). Viable cells were counted using trypan blue exclusion and then resuspended to final concentrations of 1.0 to 2.5×10^6 cells/ml in fresh PBS.

Ascorbate preloading of K562 cells

Ascorbate loading was performed according to a modification of Van Duijn and coworkers [10]. Briefly, 1.8 ml of K562 cells previously washed with cold PBS were aliquotted

Table 1
Sensitivity levels and lower limits of detection for FS, FZ, BPS, and OP

Chromogen	Extinction ($mM^{-1} \cdot cm^{-1}$) and reference	Sensitivity[a] ($mM^{-1} \cdot cm^{-1}$)	Lower limit[b] (μM)	Relative cost[c] (AU)
FS	35.5 [15]	33.2 ± 0.26	0.5 ± 0.12	1.0
FZ	27.8 [14]	25.7 ± 0.31	0.9 ± 0.15	1.0
BPS	22.2 [24]	20.7 ± 0.15	1.0 ± 0.05	2.5
OP	11.5 [19]	10.6 ± 0.02	1.4 ± 0.10	0.8[d]
FIC	1.0	1.0	—	—

[a] The stated sensitivity levels correspond to the gradients of standard curves for the respective ferrous/chromogen chelates in the presence of FOC and FIC, corrected for dilution factor and path length. Results shown are means ± SD ($n = 4$).

[b] Lower limits of detection were calculated as described in the text. Results shown are means ± SD ($n = 8$).

[c] Relative costs were determined on the basis of the price of 1 g of at least 99% purity-grade reagent from Sigma–Aldrich.

[d] The stated relative price for OP is for a unit of 5 g (the smallest unit available) from Sigma–Aldrich.