A High-Contrast Fixative for Ferricyanide Reducing Zymograms

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SUMMARY

Oxidation of sulfur by microorganisms is a topic of interest for a number of researchers worldwide. Literature reports indicate that several different pathways for sulfur oxidation may be present within a single organism. In order to discriminate between activities within these pathways, methods must be available which allow discrimination between specific sulfur oxidizing activities. We describe herein a general method of increasing contrast of ferricyanide reducing zymograms and simultaneously fixing gels for storage.

Key words: Thiobacillus, thiosulfate, Ferricyanide, ND-PAGE, zymogram.

BACKGROUND

Certain specialized microorganisms are capable of utilizing either oxidative or reductive transformations of inorganic sulfur compounds in the energy producing pathways of their metabolism. Numerous species are capable of chemolithotrophic growth using sulfur compounds as their sole energy source, with several being reported as obligate chemolithotrophs1-3). Assimilation of inorganic sulfate into biomass and subsequent recycling of biomass by microorganisms is an active process within the environment. It has been estimated that fully 75% of the sulfur in the Earth’s crust has been biologically cycled4). During aerobic chemolithotrophic growth utilizing reduced and/or partially reduced sulfur compounds, these compounds are used as electron donors with the final product of sulfur oxidation generally being sulfate (i.e., sulfuric acid). Sulfur transforming activities have been identified in numerous genera including Thiobacillus, Desulfurolobus, Paracoccus and Desulfomaculatum. These activities include: thiosulfate oxidase5,6), sulfite oxidase7,8), rhodanese9), sulfur oxygenase10), transulfurylase11) and sulfite reductase12). In addition, a thiosulfate oxidizing multienzyme system (TOMES) has been recently characterized in Paracoccus pantotrophus GB-17 that appears to oxidize thiosulfate directly to 2 moles of sulfate with no detectable free intermediates13).

Until recently, the general consensus was that there was a fundamentally common sulfur oxidation pathway operating in all of the thiobacilli and metabolically similar chemolithothrophs14). Such variations as appeared to occur in sulfur oxidation pathways of different organisms were thought to be due to differences in activity or detail of function (e.g. K_M, V_max, or enzyme concentration) of essentially common enzyme systems. However, recent successes in the preparation and partial fractionation of cell-free extracts capable of sulfur compound oxidation have indicated that different organisms may exhibit considerable diversity in the detailed molecular mechanisms for sulfur oxidation15), vide supra. In order to resolve the apparent discrepancies in sulfur oxidation mechanism between organisms, it will be necessary to have a complete understanding of the individual steps, at a molecular level, of the complete sulfur oxidation pathways within a number of organisms displaying varied activities. The Paracoccus system seems to represent a well characterized example of the multienzyme complex method of thiosulfate oxidation. We propose to use the individual enzymes of the sulfur oxidizing bacterium Halothiobacillus (Tb.) neapolitanus as a model system for the investigation of the mechanism of action of biological sulfur transformations in those microorganisms which display discrete intermediates during the oxidation of thiosulfate. We chose this species specifically because the individual steps within the overall oxidation...
scheme are reported to be independent of one another and allow individual enzyme activities to be isolated, purified and studied at the molecular level.

**MATERIALS AND METHODS**

Cytochrome c reductase (EC 1.6.99.3) and β-nicotinamide adenine dinucleotide (NADH), bovine serum albumin (BSA), diethyl aminoethyl (DEAE) SephaCel, blue-agarose, and CM-Sepharose were purchase from Sigma Chemical Co., St. Louis, MO, USA. *Halothiobacillus neapolitanus* strain 23641 was purchased from the American Type Culture Collection, Arlington, VA, USA. All other reagents were of reagent grade or better.

*Halothiobacillus neapolitanus* (ATCC 23641) was grown in a 10L chemostat utilizing thiosulfate as the sole energy source essentially as reported previously. Cells were harvested by tangential flow ultra-filtration and stored frozen at -20°C until used. Fresh frozen cells were thawed and lysed by three passes through a French press at 20,000 psi. Subsequent purification procedures were at 4°C. The lysate was centrifuged for 30 minutes at 40,000 x g and the resulting cell free extract subjected to anion-exchange, blue-dye affinity and cation-exchange chromatographies in order to purify the soluble ferricyanide dependent thiosulfate oxidizing activity. Ferricyanide dependent thiosulfate oxidizing enzyme assays were performed as previously reported by Torghrol et al. (data not shown). Thiosulfate and ferricyanide concentrations increased to 4 mM each with the modifications of Jones. Protein concentrations were estimated by the method of Bradford using BSA as a reference standard. Purified fractions containing the ferricyanide dependent thiosulfate oxidizing activity were subjected to non-denaturing polyacrylamide gel electrophoresis (ND-PAGE) by the method of Calza. Samples containing between 0.5 and 10 units of thiosulfate dependent ferricyanide reducing activity were applied to 0.7 mm × 10 cm 'mini-gels' comprised of a 1 cm 4% stacking gel and a 10% resolving gel and electrophoresed at a constant voltage of 150 V for 2.5 hours. Total protein was visualized by staining with Coomassie Brilliant Blue R-250 (data not shown). Thiosulfate oxidase enzyme activity was visualized by monitoring the thiosulfate dependent reduction of ferricyanide. The non-denaturing gel was equilibrated with 20 mM PIPES pH=6.8 buffer at 30°C. This generally required three 10 minute changes of approximately 50 ml of buffer each. The gel was then soaked for 20 minutes in approximately 50 ml of freshly made 2 mM potassium ferricyanide in PIPES pH=6.8 buffer at 30°C. The gel was briefly rinsed in PIPES buffer without ferricyanide and immediately immersed in a freshly made 20 mM PIPES buffer solution containing 2 mM sodium thiosulfate at 30°C. This solution was made fresh the day of the experiment since thiosulfate is notoriously unstable in aqueous solutions for long periods of time. The volume of this solution was approximately equal to that of the gel itself. Large solution volumes were avoided at this point since the ferricyanide is very prone to diffusion out of the gel rendering the zymogram less effective. The gel was incubated in this solution until a faint clearing zone could be visually detected. The clearing was initially detected in approximately 80–90 seconds by careful visual inspection (Fig. 1a). In order to 'fix' the gel, prevent further reduction of ferricyanide and subsequent band broadening, and enhance contrast of the developing bands of clearing, the gel was immediately immersed in an aqueous solution containing 100 mM cobalt chloride as previously described (Fig. 1b).

In order to demonstrate the utility of this method with other enzymes capable of ferricyanide reduction, a similar treatment was used for samples of cytochrome c reductase. Cytochrome c reductase was electrophoresed under conditions identical to those described for the thiosulfate oxidase until the bromophenol blue tracking dye reached the end of the gel (~1.75 hrs). The gel was then equilibrated in 20 mM Tris buffer pH=8.0 and soaked in the same buffer containing 2 mM potassium ferricyanide. The gel was then washed briefly with buffer containing no ferricyanide and immediately immersed in 20 mM Tris buffer containing 10 mM NADH. When faint zones of clearing could be detected, the gel was immersed in 100 mM cobalt chloride (Fig. 2).

**RESULTS**

The initial detection of the zone of clearing in both gels is difficult due to the poor contrast between the developing clear zone of activity and the pale yellow background of the ferricyanide containing gel (Fig. 1a). In order to achieve maximum resolution it is necessary to stop the enzyme activity at a stage when the band representing activity is as narrow as possible. Some experimentation is necessary to determine an appropriate enzyme activity loading in each lane to achieve narrow bands of activity in a reasonable time frame. In our experience, 0.5 to 20 units of activity per lane can be detected in 1 to 2 minutes of incubation time. In addition, the incubation time in substrate containing buffer must be minimized for those systems in which the product of the reaction of interest has the potential to become the substrate for other redox reactions. This is true of the system under study where thiosulfate is oxidized to tetrahionate which can subsequently disproportionate into sulfane and sulfite. The sulfite then has the opportunity to become substrate for any sulfite oxidase activity found in the sample. Both activities are known to be present in cell free extracts of a number of organisms and both can make use of ferricyanide as electron acceptor. This can make zymogram identification of thiosulfate oxidase difficult in crude enzyme preparations.
DISCUSSION

The counterstained gels clearly illustrate the advantages of using the counterstaining technique for ferricyanide reducing zymograms. The initial bands of activity are virtually undetectable because of the poor contrast (Fig. 1a). The dark background provided by the counterstain clearly shows that the intensity of the bands is concentration dependent (Fig. 1b, lanes 2-6) and activity specific (Fig. 1b, lanes 8 and 9). In summary, this technique provides a simple, cost effective method of: 1) increasing contrast on ferricyanide reducing zymograms, 2) eliminating artifacts caused by ‘downstream’ sulfur oxidizing enzyme activities in crude preparations and, 3) simultaneously providing a fixative for long term storage of Zymograms (Fig. 1c).

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REFERENCES

3) Taylor BF, Hoare DS, Hoare SL. *Thiobacillus denitrificans* as an obligate chemolithotroph. Isolation and growth