BIOCHEMISTRY OF DISSIMILATORY SULFUR OXIDATION

PROGRESS REPORT

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**Robert Blake II, Principal Investigator
Department of Biochemistry
Meharry Medical College
Nashville, Tennessee
37208**

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A. OVERVIEW OF PROJECT

A.1. We changed our experimental plan

The aims of the current proposal were to initiate the systematic identification and quantification of the relevant sulfur-transformation enzymes encoded and expressed by different species of the thiobacilli. The experimental plan had two major goals: (1) To purify known sulfur-transformation enzymes to electrophoretic homogeneity; and (2) To perform immunochemical analyses of protein expression using cell-free extracts and polyclonal antibodies directed against each protein purified in goal number one.

Reports from other laboratories caused us to rethink our strategy and deviate from our original experimental plan. The demonstration of the remarkable genotypic diversity among the thiobacilli by rRNA sequence analyses (Lane, et al., 1992, J. Bacteriol. 174, 269-278) raised valid concerns regarding the prospects for immunological cross-reactivity among enzymes with similar functions in such genetically diverse organisms. Concomitantly, reports began to appear from Kelly's and Kuenen's laboratories regarding the purification and partial characterization of sulfur transformation enzymes that appeared to represent quite different pathways for sulfur oxidation in different species of the thiobacilli. Both developments suggested that our initial plan was unlikely to significantly advance our knowledge of the enzymology of respiration on reduced sulfur compounds. We elected to temporarily abandon the immunological approach and focus instead on describing the structural and functional properties of individual purified sulfur oxidation enzymes. Toward that end, we sought to gain a level of expertise in the characterization of protein structure to complement our ongoing interests in enzyme functional studies.

Structural and steady state kinetic studies were performed on electrophoretically homogeneous adenosine 5'-phosphosulfate (APS) reductase from T. denitrificans. Steady state and rapid mixing kinetic studies were also performed on rhodanese.

We also initiated an investigation as to how a microorganism could prosper at the expense of an insoluble substrate like elemental sulfur. In the interests of developing new approaches to study the interactions of bacteria with colloidal sulfur, we acquired and performed preliminary experiments with instruments commonly employed to characterize colloidal particles.

B. SUMMARY OF PROGRESS

B.1. Structural studies on APS reductase

APS reductase was purified to electrophoretic homogeneity from cell-free extracts of T. denitrificans by a novel one-step column chromatographic separation with immobilized Cibacron Blue F3GA dye. Affinity elution of the purified protein from the dye-ligand support was accomplished with a gradient of AMP, one of the enzyme's substrates. Sodium dodecyl sulfate polyacrylamide gel electrophoresis under reducing conditions revealed that the purified protein contained both large and small subunits of approximately 70,000 and 18,000 daltons, respectively. Densitometric analyses of the Coomassie Blue-stained gels indicated that there were three large subunits for every small subunit. A more precise determination of the molecular weight of each subunit was obtained by liquid chromatography coupled with electrospray mass spectrometry in collaboration with Dr. Jack Shively at the City of Hope, Duarte, CA. Figs 1A and B show the positive-ion electrospray mass spectra of the large and small subunits, respectively, of the APS reductase. Multiply-charged ions were observed with each subunit. The respective insets show the deconvoluted spectra for the neutral polypeptides obtained using operating and analysis software provided with the mass spectrometer. Both peaks were rather broad and complex, indicating a mixture of polypeptides. The small mass differences could derive from methionine oxidation, missing N- or C-terminal amino acids due to proteolytic activity during preparation, or posttranslational modification of part of the sample.

The molecular weight of the intact enzyme was determined by Rayleigh light scattering analysis on a Dawn DSP laser photometer equipped with multiple detectors. Fig. 2 shows a Debye plot obtained with 5 concentrations of the native APS reductase. Extrapolation of the data to zero angle and concentration yielded a molecular weight of the native enzyme of around 240,000 daltons, a value very close to that anticipated for a tetramer composed of three large and one small subunits.

The enzyme readily catalyzed the oxidative condensation of sulfite and AMP to form adenosine 5'-phosphosulfate in the presence of an electron acceptor like ferricyanide. Specificity studies with numerous structural analogs of both substrates identified both alternative substrates (such as GMP for AMP) and competitive inhibitors (such as selenite for sulfite). The results of these structural and functional studies were summarized in a manuscript entitled "Respiratory enzymes of Thiobacillus denitrificans. Purification and characterization of adenosine 5'-phosphosulfate reductase" to be submitted to the Journal of Biological Chemistry. A first draft of the manuscript written by Phillip Skae in partial fulfillment of the requirements for his Ph.D. degree is currently under revision prior to submission.

Fig. 1A. Positive-ion electrospray mass spectrum of the large subunit of APS reductase. Inset, the deconvoluted spectrum showing the weight of the neutral large subunit.

Fig. 1B. Positive-ion electrospray mass spectrum of the small subunit of APS reductase. Inset, the deconvoluted spectrum showing the weight of the neutral small subunit.

Fig. 2. Debye light scattering plot for native APS reductase. Concentration of APS reductase in g/ml: closed circles, 1.15; open circles, 2.31; closed triangles, 3.46; open triangles, 4.62; and closed squares, 5.77. Measurements were performed in 0.01 M HEPES, pH 7.5. $R\theta$ is the Rayleigh ratio, θ is the angle between the incident and the scattered light, K is an instrument constant, c is the concentration of the APS reductase, and 16464 is an arbitrary constant that permits an appropriate spread of the data for plotting. The dashed lines represent extrapolation to zero angle (horizontal line) and zero concentration (vertical line).

B.2. Kinetic studies on APS reductase

The steady state kinetic mechanism of the APS reductase was determined using a combination of initial velocity, product inhibition, and dead-end inhibition kinetic experiments. The initial velocity experiments summarized in Table 1 established that the enzyme followed a Bi Uni Uni Uni Ping Pong kinetic mechanism with sulfite and AMP adding first. The product APS then dissociates, leaving two electrons behind on the enzyme-bound flavin. The oxidized electron acceptor (ferricyanide) then adds and subsequently leaves in the reduced form, completing a single catalytic cycle. Product inhibition experiments summarized in Table 2 established that the order of substrate addition was sulfite binding first followed by AMP. Dead-end inhibition experiments that exploited selenite and adenosine 5'-monosulfate (AMS) as chemically inert substrate analogs of sulfite and AMP, respectively, are summarized in Table 3. These dead-end inhibition experiments confirmed the order of substrate addition deduced in the product inhibition experiments. Substrate inhibition by both AMP and ferricyanide was observed and characterized. A manuscript summarizing these kinetic studies entitled "Steady state kinetic mechanism of adenosine 5'-phosphosulfate reductase of Thiobacillus denitrificans" was written by Phillip Skae and is currently under revision prior to submission to the Journal of Biological Chemistry.

B.3. Kinetic studies on rhodanese

An initial velocity and stopped flow spectrophotometric investigation of the sulfur-transfer reactions catalyzed by purified rhodanese was conducted with several novel chromogenic and fluorimetric substrates synthesized for the occasion. Rhodanese catalyzes the transfer of the outer sulfur atom of an organic thiosulfonate to a corresponding organic thiosulfinate, as follows:

where R can represent a hydrogen atom or any of a variety of alkyl or aryl hydrocarbons. For each thiosulfonate/thiosulfinate conjugate pair (where $\text{R}=\text{R}'$), the following kinetic experiments were performed:

a. The equilibrium constant for the interaction of each conjugate pair with the rhodanese was determined by steady state absorbance and (where applicable) fluorescence spectroscopy.

b. The individual rate constants for the transfer of sulfur from each thiosulfonate to the free enzyme and the transfer of sulfur from the enzyme-sulfur complex to each thiosulfinate were determined by stopped-flow spectrofluorometry.

Table 1. Summary of initial velocity studies with APS reductase. Reciprocal plot patterns obtained with APS reductase are compared with those predicted for 3 Ter Bi kinetic mechanisms.

Varied sub- strate	Fixed sub- strate	Predicted pattern		Ping Pong		Observed pattern
		Ordered Ter Bi	Random Ter Bi	Bi Uni	Uni Uni	
Sulfite	AMP	INT	INT	INT		INT
	Fe(CN) ₆	INT	INT	PAR		PAR
AMP	Sulfite	INT	INT	INT		INT
	Fe(CN) ₆	INT	INT	PAR		PAR
Fe(CN) ₆	AMP	INT	INT	PAR		PAR
	Sulfite	INT	INT	PAR		PAR

Table 2. Summary of product inhibition studies with APS reductase. Product inhibition patterns obtained with APS reductase are compared with those predicted for 3 Ping Pong kinetic mechanisms.

Sub- strate	Product inhi- bitor	Predicted pattern			Observed pattern
		Random addition	AMP adds first	Sulfite first	
Sulfite	APS	NC	NC	NC	NC
	Fe(CN) ₆	NC	NC	C	C
AMP	APS	NC	NC	NC	NC
	Fe(CN) ₆	NC	C	NC	NC

Table 3. Summary of dead-end inhibition studies with APS reductase. Dead-end inhibition patterns obtained with APS reductase are compared with those predicted for 3 Ping Pong kinetic mechanisms.

Sub- strate	Dead-end inhi- bitor	Predicted pattern			Observed pattern
		Random addition	AMP adds first	Sulfite first	
Sulfite	Selenite	C	C	C	C
	AMS	NC	NC	UC	UC
AMP	Selenite	NC	UC	NC	NC
	AMS	C	C	C	C
Fe(CN) ₆	Selenite	UC	UC	UC	UC
	AMS	UC	UC	UC	UC

c. Steady-state kinetic studies (using both fluorescence and absorbance spectroscopy) were conducted to show that the rate constants assigned to the transient enzyme species from the stopped-flow experiments agreed with the numerical values for the steady-state kinetic coefficients.

As a result of the kinetic experiments outlined above, a comprehensive table of equilibrium and rate data regarding rhodanese-catalyzed sulfur transfer was compiled. The Marcus theory of group transfer was successfully applied to this set of equilibria and rate data to demonstrate that the rates of sulfur transfer reactions within the active site of the enzyme were determined solely by the inherent reactivity of the sulfur donors and acceptors and that protein-dependent activation requirements exerted no influence whatsoever. A manuscript entitled "Application of Marcus theory to sulfur transfer reactions catalyzed by rhodanese" is in preparation.

B.4. Bacterial interactions with colloidal sulfur

The electrophoretic mobility of Thiobacillus ferrooxidans was determined by laser Doppler velocimetry under physiological conditions in the absence and presence of colloidal sulfur. Colloidal sulfur was negatively charged at pH 2.0 in sulfuric acid. When grown on elemental sulfur, washed cells were close to their isoelectric point under the same conditions. The electrical double layer around the bacterial cells under physiological conditions thus exerted minimal electrostatic repulsion in possible interactions between the cell and the negatively charged insoluble sulfur. When T. ferrooxidans was mixed with colloidal sulfur at pH 2.0, the mobility spectra of the free components disappeared with time to be replaced with a new colloidal particle whose electrophoretic properties were intermediate between those of the starting components. This new particle had the charge and size properties anticipated for a complex between the bacterium and its insoluble substrate. The utility of such measurements to study the interactions of these chemolithotrophic bacteria with their insoluble sulfur substrate was demonstrated. These experiments were summarized as part of a manuscript entitled "On the solubilization of minerals by bacteria. Electrophoretic mobility of Thiobacillus ferrooxidans in the presence of iron, pyrite, and sulfur" recently submitted to Applied and Environmental Microbiology. A copy of the manuscript is attached.