MECHANISM OF THIOSULFATE OXIDATION BY A FACULTATIVE AUTOTROPH *THIOBACILLUS NOVELLUS*

by

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A dissertation submitted

in partial fulfillment of the requirements

for the degree of Doctor of Philosophy in the

University of Manitoba

1966

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ACKNOWLEDGEMENTS

The author wishes to express with sincerest gratitude and appreciation his indebtedness to Dr. Isamu Suzuki for his invaluable assistance and suggestions, and above all his enthusiasm and encouragement throughout the course of this investigation, and in the preparation of the manuscript.

The author also wishes to acknowledge his indebtedness to Dr. H. Lees who introduced him to autotrophy and whose continued interest was gratefully appreciated.

ABSTRACT

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The mechanism of carbon dioxide fixation by Thiobacillus novellus was studied with $C^{14}O_2$ using both whole cells and cell-free extracts. Under autotrophic conditions with thiosulfate as substrate the organism fixed CO_2 through the carboxydismutase pathway and the phosphoenolpyruvate pathway in accordance with the mechanisms found in other autotrophs. Under heterotrophic conditions with glucose as substrate, however, the carboxydismutase pathway was repressed and the phosphoenolpyruvate pathway was the only pathway found.

Both whole cells and extracts of T. novellus oxidized thiosulfate, sulfur, sulfite and sulfide, but tetrathionate was oxidized only by whole cells. The sulfur-oxidizing enzyme was found to be similar to the enzyme found in other thiobacilli requiring reduced glutathione as cofactor. Sulfide was oxidized with an intermediary formation of sulfur. Sulfite was oxidized through a cytochrome system involving sulfite:cytochrome c oxidoreductase, cytochrome c and cytochrome oxidase. Rhodanese activity was found in extracts of this organism. A mechanism of thiosulfate oxidation was proposed where thiosulfate is initially cleaved to sulfur and sulfite by a rhodanese-like enzyme, sulfur is then oxidized to sulfite by the sulfur-oxidizing enzyme, and finally sulfite is oxidized to sulfate through a cytochrome system.

Sulfite:cytochrome c oxidoreductase was partially purified and its properties were studied. It was found to be a new enzyme distinct from APS-reductase of Thiobacillus thioparus. The enzyme reduced

ferricyanide or cytochrome c with sulfite stoichiometrically and was specific for sulfite as substrate. The enzyme was inhibited by sulfhydryl inhibitors and various monovalent anions. The inhibition by NaCl was competitive with respect to sulfite concentrations. The K_m value for sulfite was found to be 4×10^{-5}M at pH 8.0 and 2×10^{-6}M at pH 6.5. The activity, however, was much higher at pH 8.0. The significance of these findings is discussed in relation to the mechanism of sulfite and thiosulfate oxidations by T. novellus.

Oxidative phosphorylation was shown in extracts of T. novellus during sulfite oxidation and was concluded to be the mechanism of energy generation in this organism rather than the substrate-level phosphorylation mechanism proposed for T. thioparus.

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ABBREVIATIONS

ADP	-	adenosine-5'-diphosphate
AMP	-	adenosine-5'-phosphate
APS	-	adenosine-5'-phosphosulfate
ATP	-	adenosine-5'-triphosphate
Cyt	-	cytochrome
DEAE	-	diethylaminoethane
DNP	-	2, 4 dinitrophenol
EDTA	-	ethylenediaminetetracetate
FDP	-	fructose-1,6-diphosphate
F6P	-	fructose-6-phosphate
GSH	-	reduced glutathione
G6P	-	glucose-6-phosphate
NAD	-	oxidized nicotinamide adenine dinucleotide
NADP	-	oxidized nicotinamide adenine dinucleotide phosphate
PEP	-	phosphoenolpyruvate
3-PGA	-	3-phosphoglyceric acid
RDP	-	ribulose-1,5-diphosphate
R5P	-	ribose-5-phosphate
Ru5P	-	ribulose-5-phosphate
TCA	-	trichloracetic acid
Tris	-	tris (hydroxymethyl) aminoethane

INTRODUCTION

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The thiobacilli hold a unique position among the non-photosynthetic bacteria since some members are strict autotrophs whereas others are facultative, i.e., they are capable of either autotrophic or heterotrophic growth. Autotrophically grown thiobacilli utilize either thiosulfate or sulfur as energy-source, but there is general disagreement as to the end-products formed, and only the report of Peck (1960) and Peck and Fisher (1962) have substantiated their results by enzymatic evidence.

Since both whole cells and extracts of T. novellus actively oxidized thiosulfate to sulfate without accumulation of polythionates and the extracts had both the sulfur- and sulfite-oxidizing systems, thiosulfate oxidation by this organism was investigated in order to elucidate the enzymatic mechanisms involved. Initial studies of the oxidation of sulfite revealed that AMP was not stimulatory. The enzyme responsible was subsequently purified and found to be sulfite oxidase rather than APS-reductase. During purification of the sulfite oxidizing enzyme, an enzyme which oxidized elemental sulfur to thiosulfate was also isolated and found to be similar to that observed in T. thiooxidans (Suzuki 1965) and T. thioparus (Suzuki and Silver 1966). Since sulfur and sulfite were intermediates of thiosulfate metabolism, it was believed that a scission of thiosulfate was the initial reaction. The enzyme responsible was subsequently found to be rhodanese.

Since T. novellus does not metabolize thiosulfate according to the pathway involving substrate-level phosphorylation reactions, the possibility of oxidative phosphorylation was studied. Oxidative phosphorylation was found to be the mechanism for deriving energy in this organism. The pathway of CO_2 fixation was also investigated in order to compare the mechanism of T. novellus with that of the obligately autotrophic thiobacilli. The results indicated that the synthesis of cellular carbon proceeded by way of the 3-PGA pathway and the PEP carboxylase system in accordance with the mechanism in other thiobacilli.

It is hoped that as a result of the findings of this investigation a better understanding of the metabolism of reduced sulfur compounds and of CO_2 by this organism will be obtained, and that some of the discrepancies of other investigations will be resolved.

HISTORICAL

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Winogradsky (1887) established the fundamental principles of autotrophy among bacteria, when he concluded from studies of certain sulfur- and iron-oxidizing organisms that all their energy requirements were derived from the oxidation of incompletely oxidized inorganic compounds and all of the carbon by the fixation of CO_2. His experiments were conducted with members of the genus Beggiatoa which recently have been found to be unable to grow on the energy released from sulfur, and are heterotrophic (Starkey, 1962). Thus whereas the concepts of autotrophy were correct, the organisms from which they were drawn did not live autotrophically as they were believed to.

Among the autotrophic bacteria are those which metabolize reduced inorganic sulfur compounds to sulfate and are classified in the genus Thiobacillus (Breed et al., 1957). In this genus are found both strictly autotrophic and facultatively autotrophic species. This discussion will be concerned with the physiology of Thiobacillus novellus, including appropriate information about the metabolism of other species of the genus. Some aspects of the metabolism of carbon compounds will also be presented. For reviews on the thiobacilli, C. B. Van Niel (1954), Lees (1955), and Vishniac and Santer (1957) should be consulted. The mechanisms of autotrophic carbon dioxide fixation, are well documented in the report of Elsden (1962), while those of heterotrophic CO_2 assimilation are found in the report of Wood and Stjernholm (1962). Because of these extensive reviews,

details will be treated very briefly here.

General physiology of Thiobacillus novellus

Starkey (1935) isolated a small, non-motile, gram-negative, non-sporulating rod, which grew on organic as well as inorganic carbon sources. When grown with CO_2 as carbon source, it utilized thiosulfate as a source of energy and was therefore a facultative autotroph. This organism was named Thiobacillus novellus. On an inorganic salt medium containing thiosulfate, the acidity of the medium was increased as growth progressed (Starkey, 1934[a]). Since the organism developed best at pH between 8.0 and 9.0, it somewhat resembled Thiobacillus thioparus which developed best at pH close to neutral except that the latter was motile and accumulated sulfur during thiosulfate oxidation. These two organisms were unlike Thiobacillus thiooxidans (Waksman and Joffe, 1921) which also oxidized thiosulfate or elemental sulfur, but developed best in an acid environment (Starkey, 1934[b]). On the basis of these differences T. novellus and T. thioparus are considered as alkaline thiosulfate oxidizers, and T. thiooxidans as acid sulfur oxidizer (Umbreit, 1962). Among the facultative thiobacilli are T. novellus, the best known of the group, T. coproliticus, isolated from a piece of Triassic coprolite (Lipman and McLees 1940), and T. intermedius, the most recently isolated member of the genus (London, 1963).

Although externally supplied organic compounds do not support growth of the obligately autotrophic thiobacilli in the absence of

sulfur compounds, at least some such compounds penetrate into the cell. Waksman and Starkey (1922) have found that the rate of sulfur oxidation by T. thiooxidans increased slightly in the presence of glucose. Glucose slowly disappeared during sulfur oxidation and its consumption was proportional to the growth of the organism (Starkey, 1925).

Suzuki (1958) incubated T. thiooxidans whole cells and extracts with C^{14}-labelled glucose and found $C^{14}O_2$, as well as labelled amino acids, sugars and polysaccharides. In view of these findings, obligate autotrophy cannot be explained in terms of cell-walls or membranes impermeable to organic matter. The difference between obligate and facultative thiobacilli probably lies in the inability of the former to generate sufficient amounts of energy for growth from the metabolism of organic compounds, and may be quantitative rather than qualitative.

Mechanism of carbon dioxide fixation

The autotrophic mechanism of carbon dioxide fixation in bacteria is the same as that found by Calvin and his associates using C^{14}-labelled CO_2 during investigations of a photosynthetic alga. In this mechanism CO_2 is directly involved in only one reaction, namely, the formation of 3-phosphoglyceric acid (3-PGA) which was labelled in the carboxyl group and was the earliest stable product detected in the experiments (Calvin and Benson, 1948; Bassham et al., 1950;

Benson et al., 1950). The 3-PGA arose from a condensation of $C^{14}O_2$ with ribulose-1,5-diphosphate (RDP) (Calvin and Massini, 1952; and Bassham et al., 1954). The enzyme catalyzing this reaction was termed RDP carboxylase or carboxydismutase. (Weissbach and Horecker, 1956).

The RDP arose from ribulose-5-phosphate (Ru5P) and adenosine-5'-triphosphate (ATP) by a reaction catalyzed by phosphoribulokinase (Hurwitz et al., 1956). Ru5P could be replaced by ribose-5-phosphate (R5P) in the presence of phosphoriboisomerase.

PGA is converted to hexose primarily by a reversal of the Embden-Meyerhof pathway, being first reduced to triose phosphate, which is then converted to fructose-1,6-diphosphate (FDP) by aldolase, fructose-6-phosphate (F6P) arising by phosphate removal of FDP.

Pentose phosphates are regenerated by the action of transketolase and transaldolase, enzymes commonly involved in carbohydrate metabolism. Thus from the evidence available, only two enzymes unique to the autotrophic mechanism of CO_2 fixation are involved, namely, carboxydismutase catalyzing the condensation reaction between CO_2 and RDP, and phosphoribulokinase forming RDP from Ru5P and ATP.

The mechanism of CO_2 fixation in chemosynthetic bacteria is well documented in the literature, and its occurrence by way of the RDP carboxylating mechanism was found in T. thioparus (Santer and Vishniac, 1955), Thiobacillus denitrificans (Trudinger, 1955, 1956; Milhaud et al., 1956) and T. thiooxidans (Suzuki and Werkman, 1958[a]).

Although there is overwhelming support for the 3-PGA pathway as

a means of CO_2 fixation in autotrophs, it should be pointed out that other mechanisms exist for the incorporation of CO_2 into organic carbon. The Wood-Werkman reaction (Wood and Werkman, 1938) is essential for the operation of the tricarboxylic acid cycle and for the biosynthesis of various amino acids. Utter and Kurahashi (1953, 1954[a,b]) isolated oxalacetic carboxylase from liver. The enzyme catalyzed a reversible reaction between phosphoenolpyruvate (PEP), CO_2 and oxalacetate requiring inosine or guanosine diphosphate as phosphate acceptor. Phosphoenolpyruvate carboxylase (PEP carboxylase) isolated from spinach leaves (Bandurski and Greiner, 1953) catalyzed the formation of oxalacetate and orthophosphate from PEP and CO_2 in an irreversible reaction. Both of these enzymes were present in T. thiooxidans (Suzuki and Werkman, 1957, 1958[a]) and were apparently responsible for the rapid labelling of the β-carboxyl group of aspartate and γ-carboxyl group of glutamate in the whole cell experiments (Suzuki and Werkman, 1958[b]). The carboxydismutase activity of T. novellus was dependent upon growth conditions since in cells grown on organic media the enzyme level fell to less than 2% of the autotrophic level (Vishniac and Trudinger, 1962). Similar results were also obtained in an extension of these studies with the same organism (Aleem, 1965).

Oxidation of sulfur compounds

The thiobacilli are a small group of microorganisms capable of obtaining all the energy required for growth from the oxidation of

reduced inorganic sulfur compounds to sulfate. In spite of the wealth of literature available on the oxidation of sulfur compounds by thiobacilli, the mechanism whereby this is accomplished still is a controversial topic.

When grown on thiosulfate, some thiobacilli produced tetrathionate along with sulfur and sulfate (Gleen and Quastel, 1953; Vishniac, 1952; Jones and Happold, 1961; Vishniac and Trudinger, 1962), while others produced only sulfur and sulfate (Starkey, 1935; Parker and Prisk, 1953; Peck, 1960). The variation in end-products obtained by different workers has been interpreted as being due to growth conditions as well as the organisms investigated (Peck, 1962). According to Starkey (1934[a,b]) and Parker and Prisk (1953) T. novellus did not produce tetrathionate during growth on thiosulfate. Thiosulfate was oxidized according to the overall equation:

$$Na_2S_2O_3 + 2O_2 + H_2O \longrightarrow Na_2SO_4 + H_2SO_4$$

Variations in the results of growth experiments have resulted in widely divergent views on the mechanism of thiosulfate oxidation. Vishniac and Santer (1957), based on the observation (Vishniac, 1952) that tetrathionate was produced during thiosulfate oxidation of resting cells of T. thioparus, have suggested a transformation of the sulfur atoms of thiosulfate by way of the formation of several polythionates. Further support for this hypothesis came from the isolation of a soluble enzyme from Thiobacillus X (Trudinger, 1961) which catalyzed the quantitative conversion of thiosulfate to tetra-

thionate. A similar enzyme was obtained by Santer while investigating T. thioparus and autotrophic T. novellus (Trudinger and Vishniac, 1962). London and Rittenberg (1964) demonstrated the accumulation of polythionates during thiosulfate oxidation by cell-free extracts of T. thioparus and T. thiooxidans and the oxidation of tetrathionate by the same extracts. This pathway involving polythionates as intermediates between thiosulfate and sulfate still suffers from the lack of knowledge of specific enzymes responsible for the overall oxidation of thiosulfate to sulfate.

Skarzyński et al., (1957), on the basis of growth experiments with S^{35}-labelled thiosulfate ($S\text{-}SO_3^{2-}$), have concluded that thiosulfate scission occurs at the cell-membrane and the outer sulfur (S-) is the only part of the molecule that enters the cell and is metabolized. Peck (1960) proposed a similar mechanism based on studies of thiosulfate oxidation by extracts of T. thioparus and suggested that the initial reaction was the reduction of thiosulfate to sulfide and sulfite. Sulfide was converted to sulfur and sulfite eventually to sulfate. The enzyme specific for each reaction was also shown and is outlined in the following equations:

$$2S_2O_3^{2-} + 4H^+ + 4e^- \xrightarrow[\text{reductase}]{\text{thiosulfate}} 2SO_3^{2-} + 2H_2S$$

$$2H_2S + O_2 \xrightarrow[\text{oxidase}]{\text{sulfide}} 2S^\circ + 2H_2O$$