OXIDATIVE PHOSPHORYLATION IN THIOBACILLUS NOVELLUS

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SUMMARY

A soluble cell-free fraction(144,000xg supernatent) catalyzed phosphorylation coupled to the oxidation of thiosulfate, succinate and ascorbate yielding P/O ratios of 0.96, 1.9, and 0.76 respectively. However with NADH as the electron donor, the P/O ratios were very low and ranged between 0.2 - 0.3. In all cases the phosphate esterification was completely uncoupled by 2,4-dinitrophenol, m-chlorocarbonyl cyanide phenylhydrazone, 2,6-dibromophenol or pentachlorophenol. The non-particulate nature of the cell-free preparations catalyzing oxidative phosphorylation was established by electron microscopic examination.

In the facultative chemoautotroph Thiobacillus novellus the thiosulfate oxidase activity is localized chiefly in the cell-free supernatent fraction obtained by differential centrifugation of the crude cell-free extracts at 144,000xg for 60 min (1). This fraction has been shown to catalyze the enzymatic transfer of electrons from thiosulfate to molecular oxygen involving cytochromes of c, a, and o type.

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In addition, the same soluble fraction is able to catalyze an energy-dependent reversal of electron transfer from ferrocytochrome c to NAD^+ when either thiosulfate or succinate serve as electron donors; in this system the energy requirement can be met by either exogenously added ATP or by the aerobic oxidation of thiosulfate or succinate (2,3). These observations thus suggest that the soluble cell-free extracts from T. novellus possess the enzymes as well as the electron transport carriers required for the energy generation and energy utilization processes. Experiments were undertaken therefore, to investigate the ability of the non-particulate cell-free preparations to catalyze the process of oxidative phosphorylation coupled to the oxidation of thiosulfate or organic oxidizable substrates.

MATERIALS AND METHODS

Thiobacillus novellus was grown autotrophically on thiosulfate, and heterotrophically on succinate or glutamate, as described previously (1). Cells were harvested in a De-Laval Gyrotest continuous flow centrifuge, washed once with Tris-HCl buffer (0.05 M, pH 7.2) and suspended in sonication medium containing 0.3 M sucrose, 0.5 mM EDTA, 0.5 mM reduced Glutathione, and 1 mM $MgCl_2$ in 0.05 M Tris-HCl (pH 7.5). Cells were disrupted by sonication at 3^o for 3 min at 30 sec intervals in a rosette cooling-cell immersed in a salt-ice bath and using maximum power output in a Model-W-185-D Sonifier(Heat Systems Co.). The crude extracts were

centrifuged for 30 minutes at 30,000xg. The supernatent was further centrifuged in a Beckman L-2 preparative ultra-centrifuge at 144,000xg for 1 hr. Oxygen consumption was measured by a Y.S.I. oxygen polarograph (Yellow Springs Instrument Co.). In order to minimize endogenous oxygen uptake the enzyme was diluted with 0.05 M Tris-HCl pH 7.5 (final protein concn = 2 mg/ml).

Samples for ATP measurement were taken at various time intervals after substrate addition and deproteinized according to the procedure of Gibson and Morita (4). The amount of ATP formed was measured by the Luciferin-Luciferase assay procedure described by Strehler(5). Experimental details are provided in the legend to tables. Samples of the enzyme preparations were examined in a Hitachi Electron Microscope (Model HU-11A) using low angle shadowing.

Glutathione and ADP were obtained from Sigma Chemical Co. and other chemicals were of reagent grade.

RESULTS AND DISCUSSION

The cell-free fraction 144,000xg supernatent obtained from autotrophically grown cells catalyzed the enzymatic oxidation of thiosulfate or ascorbate; this process was coupled to ATP synthesis and yielded P/O ratios approaching 1.0 (Table 1). Since both thiosulfate and ascorbate enter the electron transport chain at the cytochrome *c* level, the results suggest that the terminal site of energy conservation involving the action of cytochrome *c*:O_2 oxidoreductase was functional in the chemoautotroph. Although the enzyme pre-

TABLE I

Oxidative Phosphorylation Catalyzed by Cell-Free Fraction 144,000xg Supernatent from Autotrophically Grown T. novellus

Substrate	ATP formed (nmoles)	O_2 consumed (natoms)	P/O (corrected)
Thiosulfate	57	60	0.96
Ascorbate	91	120	0.76
Succinate	306	160	1.91
NADH	83	330	0.25

Reaction mixture in a total volume of 3.0 ml contained 10 mM substrate, 1.7 mM ADP, 3.0 mM phosphate (pH 7.5), 3.0 mM $MgCl_2$, 10 mM KF, 200 µmoles of Tris-HCl buffer (pH 7.5) and cell free extract containing 6.0 mg of protein. Reaction vessel was shaken at 30° and the experiment was terminated after 3 min. Oxygen uptake was measured polarographically in identical reaction mixtures. The P/O ratios were corrected for endogenous ATP synthesis and O_2 uptake. The P/O ratios ranged between 0.56-1.0 for thiosulfate, 0.5-0.8 for ascorbate, 1.3-2.0 for succinate, and 0.2-0.3 for NADH.

In the absence of KF a potent ATPase was found in the soluble system hydrolyzing 25 nmoles of ATP/mg/min. No ATPase was observed in the KF treated system, however the uncoupled system hydrolyzed 0.78 nmoles of ATP/mg/min.

parations lacked respiratory control, it is interesting indeed that the efficiency of energy conservation by the soluble cytochrome oxidase system in T. novellus approached that of the mitochondrial preparations. This high efficiency of energy conservation was also exhibited by succinate oxidation and coupled phosphorylation; in the latter case the P/O ratios approached 2.0. It may therefore be concluded that the energy-

coupling site between cytochrome b and cytochrome c was also operative. The oxidation of exogenously added NADH was coupled to ATP formation but the P/O ratios were always very low and ranged between 0.25 to 0.3. Curiously enough, the addition of particles to the 144,000xg supernatent had no effect on the overall P/O ratios obtained with NADH oxidation. We have also observed that the cell-free extracts obtained from T. novellus grown on glutamate or succinate lacked thiosulfate oxidase but yielded essentially similar P/O ratios upon oxidation of succinate or ascorbate; however, very low P/O ratios were again obtained when NADH served as the electron donor.

The reason for the low efficiency of energy conservation coupled to NADH oxidation is not clear at present. It may be that the exogenously added NADH is unable to couple with the endogenous pyridine nucleotide and therefore, the bulk of the added NADH is oxidized via a nonphosphorylating pathway.

The effect of uncoupling agents is shown in Table II. The data clearly indicate that phosphorylation coupled to the oxidation of thiosulfate, ascorbate, succinate, and NADH is completely inhibited by 2,4-dinitrophenol(DNP), m-chlorocarbonyl cyanide phenylhydrazone(CCCP), 2,6-dibromophenol(DBP), and pentachlorophenol(PCP) while the oxygen consumption in the presence of these uncouplers was either unaffected or slightly decreased. In addition, Oligo-

TABLE II

Effect of Uncouplers on Oxidative Phosphorylation in *T. novellus*

Substrate	Uncoupler	Conc. (mM)	O_2 consumed (natoms)	ATP formed (nmoles)	P/O corrected
Thiosulfate	None	--	60	57	0.96
	DNP	0.10	60	0	-
	CCCP	0.02	60	0	-
	DBP	0.30	52	0	-
	PCP	0.10	54	0	-
Ascorbate	None	--	120	91	0.76
	DNP	0.10	84	0	-
	CCCP	0.02	86	0	-
	DBP	0.30	72	0	-
	PCP	0.10	72	0	-
Succinate	None	--	160	306	1.91
	DNP	0.10	160	45	0.30
	CCCP	0.02	160	0	-
	DBP	0.30	136	0	-
	PCP	0.10	96	0	-
NADH	None	--	330	83	0.25
	DNP	0.10	309	0	-
	CCCP	0.02	330	0	-
	DBP	0.30	281	0	-
	PCP	0.10	278	0	-

Experimental conditions were the same as described in Table I except that various uncouplers were added at concentrations shown.

mycin at 0.5 µg/mg of enzyme protein completely inhibited phosphorylation without affecting oxygen uptake.

Attempts to demonstrate oxidative phosphorylation coupled to thiosulfate oxidation in thiobacilli have not been very successful except in the case of *Thiobacillus neopolitanus* (6). Davis and Johnson (7), however, were able to demonstrate

DNP-sensitive and insensitive phosphate esterification coupled to sulfite oxidation in T. thioparus but the P/O ratios were 0.13. Likewise Charles and Suzuki (8) reported low P/O ratios of 0.1 with sulfite oxidation in T. novellus, however, the phosphorylation was inhibited only by 30% in the presence of 0.5 mM DNP.

In this report we have shown conclusively that in T. novellus, unlike most other biological systems, the process of oxidative phosphorylation coupled either to the oxidation of thiosulfate or of organic electron donors, is associated with the non-particulate cell-free preparations and that this process is also uncoupled by the classical uncoupling agents. Although Pinchot (9,10,11) has shown a soluble intermediate of oxidative phosphorylation released from phosphorylating particles of Alcaligenes fecalis, to our knowledge this is the first published report of oxidative phosphorylation in a soluble system.

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