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5.1 MATERIALS

5.1.1 General Chemicals

Sucrose, CaCl₂·2H₂O, CuSO₄·2H₂O, FeSO₄·7H₂O, MgSO₄·7H₂O, sodium succinate, sodium chloride, formic acid and H₂SO₄ were of AnalaR grade from BDH, India. Ethylenediaminetetraacetate (EDTA), Na₂HPO₄, K₂HPO₄ and KH₂PO₄ were from E.Merck, Germany (Für Analyse). Ferricytochrome c, xanthine, xanthine oxidase, bovine serum albumin and methyl viologen were obtained from Sigma Chemical Company, USA.

5.2 CULTURE MEDIUM, ORGANISM AND MAINTENANCE OF STOCK

Burk’s nitrogen free medium described in 2.1.4 was used for the growth of the bacterial cultures. *A. vinelandii* was used in these studies. Maintenance of these bacteria is described in Section 2.1.3.

5.3 METHODS

5.3.1 Bacterial Growth Under Methyl Viologen Stress

Fresh stationary *A. vinelandii* cells (0.1ml) were inoculated into 25 ml of Burk’s nitrogen free medium and shaken at 30°C in a waterbath at a speed of 160 rpm. The medium contained 0, 20, 50 and 80 μM of methyl viologen. The growth of bacteria was monitored every two hours for 24 hours by taking turbidimetric measurements at 510 nm using a spectrophotometer (Spectronic-20, Bausch and Lomb, USA). Generation time was calculated as shown in Section 2.2.1.
5.3.2 Assay of Superoxide Dismutase Under Various Stress Conditions

5.3.2.1 Preparation of assay sample

The method adopted for sample preparation was essentially that of Stowers and Elkman (1981). The cell pellet was suspended in 50 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM EDTA. The cells were sonicated in ice using an ultrasonic Soniprep-150 sonicator (15 sec. burst with 15 sec. rest, 8 cycles). The unbroken cells were removed by centrifugation in an Eppendorf centrifuge at 4°C and 10000xg. The supernatant was collected and used for SOD assay.

5.3.2.2 SOD assay

SOD was assayed essentially as per McCord and Fridovich (1969). The following reagents were added to a quartz cuvette with 1 cm light path to give a final volume of 2.8 ml. The assay mixture contained \(1 \times 10^{-5}\) M ferricytochrome c, \(8 \times 10^{-5}\) M xanthine and sufficient xanthine oxidase to produce a rate of reduction of ferricytochrome c at 550 nm by 0.025 absorbance unit (O.D.)/minute. To this reaction mixture, 100 μl of enzyme preparation (sample) was added immediately and the change in absorbance at 550 nm was noted after every 15 sec. in Shimadzu UV 240 spectrophotometer.
SOD inhibits reduction of ferricytochrome c by xanthine-xanthine oxidase system. The amount of SOD required to inhibit the rate of reduction of cytochrome c by 50% (i.e. 50% of 0.025 absorbance unit per minute is defined as one unit of SOD activity).

5.3.2.3 Methyl viologen treatment

Bacterial cells were grown with different concentrations of methyl viologen as shown in Section 5.3.1. Cells not treated with methyl viologen were used as control. Cells grown for different periods were harvested (5000xg, 8 min, 4°C) in a Sorvall high speed refrigerated centrifuge. The pellets were washed twice with 50 mM potassium phosphate buffer (pH 7.8). The samples were prepared as shown in Section 5.3.2.1 for SOD assay.

5.3.3 Nitrogenase Assay Under Methyl Viologen Stress

Bacterial cultures grown in the presence of three different concentrations of methyl viologen as shown in Section 5.3.1 were used for nitrogenase assay. Untreated cells were used as control. Nitrogenase assay methodology is given in Section 2.2.5 of Part A.

5.3.4 Experiments on Respiratory Chain

5.3.4.1 Preparation of cell suspension

Ten millilitre of A. vinelandii culture grown till stationary phase was inoculated into 1000 ml of Burk’s nitrogen free medium in 5 l flasks. Six litres of such cultures were grown aerobically at 30°C for 44h with vigorous shaking in a gyratory incubator
shaker (New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.) till the stationary phase was reached. The cells were harvested at 5000 xg using Sorvall RC-5B refrigerated superspeed centrifuge. The cells were washed with cold 25 mM sodium potassium phosphate (25 mM Na₂HPO₄, 25 mM KH₂PO₄, pH 7.4; NKP) buffer and resuspended in the same buffer.

5.3.4.2 Preparation of sub-cellular fraction

The suspension of the whole cells was disrupted by passage through a French pressure cell (SLM Instruments Inc, Urbana, Illinois, USA) twice at 13000 lb/in². Cellular debris and unbroken cells were removed by centrifugation at 10000 xg for 15 minutes at 5°C in a Beckman Model J2-21 centrifuge. The supernatant (cell free extract) was carefully removed and further centrifuged at 35000xg for 30 minutes in Beckman L8-55 M ultracentrifuge. The pellet was discarded and the slightly turbid reddish-brown supernatant was once more centrifuged at 105000 xg for 90 minutes to yield the tan coloured small particles (membrane/subcellular fraction) which were washed with NKP buffer and used for the study of cytochromes. The preparation of the subcellular/membrane fraction was carried out at 0-5°C. Protein content of this fraction was estimated by Lowry’s method as described in Section 2.2.6.

5.3.4.3 Difference spectroscopy

5.3.4.3.1 Wavelength scanning

Spectral analyses were performed with a Hitachi (model UV-2000) recording spectrophotometer using 600 µl capacity cuvettes having 1 cm path length. Sodium succinate was used as a reducing agent.
For studies on c type cytochromes (cytochromes $c_4 + c_5$) sample amounting to 5 mg protein was taken in both reference and sample cuvettes. NKP buffer was added to make up to 500 µl in each cuvette. To the sample cuvette sodium succinate was added to give a final concentration of 9 µM. The reference cuvette was kept in the oxidized state by shaking it gently once or twice. The sample was scanned between 500 and 600 nm. This formed the control experiment. In another set of experiments, $O_2^-$ were introduced into the sample cuvette containing sodium succinate by the addition of methyl viologen to a final concentration of 9 µM prior to scanning.

For studies on cytochrome o, 5mg protein equivalent samples were taken in both the cuvettes and the volume was made up to 500 µl with NKP buffer. Sodium succinate (9 µM final concentration) was added to both the cuvettes. The sample cuvette was bubbled with CO for 2 minutes. The sample was then scanned from 500 to 600 nm. In yet another experiment, the sample cuvette contained 9 µM of methyl viologen as additional reagent. The rest of the experimental procedures were as mentioned above.

5.3.4.3.2 Time scanning (reduction kinetics)

Reduction kinetics of cytochromes $c_4 + c_5$ and $b_1$ were studied using Hitachi UV-2000 recording spectrophotometer as described above. All the measurements were carried out in a total volume of 500 µl. The reaction mixture contained NKP buffer and 5 mg protein sample. The reaction was started by adding 9 µM sodium succinate to the sample cuvette while the reference cuvette was kept in the oxidized state by shaking it gently once or twice. The rate of reduction of cytochromes $c_4 + c_5$ and $b_1$ was monitored at 551 and 560 nm as a function of time. Similar experiment was also
performed by adding 9 µM methyl viologen to the sample cuvette which already contained 9 µM of succinate.

5.3.4.4 Kinetics of oxygen consumption

Kinetics of oxygen consumption were determined using oxygen electrode (Systronics, India). The same reaction mixture as mentioned under the previous Section 5.3.4.3.2 was used. The total volume of the reaction mixture was 1 ml. The reaction was started by adding 9 µM of succinate to the electrode chamber and the rate of oxidation of succinate was monitored until the chamber became anaerobic. In a second set of experiments, $\text{O}_2^-$ generating system (9 µM final concentration of methyl viologen) was added to the electrode chamber and the rate of oxidation of succinate was studied. For inhibition studies, various amount (0 to 4mM final concentration) of potassium cyanide was injected to the electrode chamber and the rate of the reaction (oxidation of succinate) was followed. Similar experiment was performed during the generation of $\text{O}_2^-$. All the experiments were conducted at room temperature.