The 503nm Pigment of *Escherichia coli*

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The yield of cell protein was one-third less for streptomycin-dependent *Escherichia coli* B than for the wild-type parent strain when both were grown aerobically on a medium with limiting glucose, but anaerobically the yield of protein was similar for both strains. The transient pigment absorbing at 503nm that is known to be present in *E. coli* and other organisms was not detectable in streptomycin-dependent mutants nor in a non-dependent (energy-deficient) revertant. When wild-type *E. coli* B was grown on limiting glucose-salts medium containing 2,4-dinitrophenol, the yield of cell protein was decreased and formation of the 503nm pigment was inhibited. Fumarase, aconitase and glucose 6-phosphate dehydrogenase were de-repressed in *E. coli* B cells grown with excess of glucose in a medium containing 2,4-dinitrophenol. In air-oxidized, wild-type *E. coli* B cells, the 503nm pigment appeared before reduced cytochromes when gluconate was the substrate but failed to appear when succinate was the substrate. The results provide evidence for a role of the 503nm pigment in aerobic energy metabolism, possibly as an electron acceptor from NADPH.

Several investigators have reported the occurrence of a pigment absorbing at 503nm in micro-organisms, e.g. *Saccharomyces cerevisiae*, *Escherichia coli*, *Bacillus megaterium* (see Lindenmayer & Smith, 1964) and *Azotobacter vinelandii* (Goucher & Kocholaty, 1957). However, because of the transient, unstable nature of the pigment, it has not been isolated nor has its structure or function been established. Lindenmayer & Smith (1964) reported that although the 503nm pigment appeared in whole yeast cells, it disappeared on cell rupture. They observed that the extinction at 503nm changed on exhaustion or addition of oxygen; however, since these changes were much slower than those of the respiratory pigments, the 503nm component was thought to be involved in other reactions. From the position of the peak and its reactivity, Lindenmayer & Smith (1964) suggested that it could be the semiquinone form of ubiquinone. Labbe, Volland & Chaix (1967) found the 503nm pigment to be similar to protoporphomethene, which they prepared from protoporphyrin.

In studies of difference spectra of whole cells of wild-type *E. coli* B, we have observed the 503nm pigment as a large symmetrical peak, together with the cytochromes absorbing at 560, 600, 620 and the flavin trough at 460nm. The absence of the 503nm peak from a streptomycin-dependent mutant of *E. coli* B, having impaired energy metabolism, prompted the present investigation.

METHODS AND MATERIALS

Organisms. Two wild-type strains of *E. coli* were used, *E. coli* B (A.T.C.C. 11303) and *E. coli* K-12. The corresponding streptomycin-dependent mutants were isolated as described by Coukell & Polglase (1965). *E. coli* B (R4) is a streptomycin-sensitive revertant obtained from streptomycin-dependent *E. coli* B.

Culture conditions. (a) Growth of cells for difference spectra. A minimal salts medium, pH 7.0, as described by Davis & Mingioli (1950) but without citrate, was used for all cultures. The carbon source (glucose), autoclaved separately in concentrated solution, was added to the medium to obtain the final concentration required. Streptomycin-dependent strains were grown on medium supplemented with 1 mg of dihydrostreptomycin (Merck, Sharp and Dohme, Montreal, Que., Canada)/ml.

A culture of cells, grown overnight in a 37°C shaking water bath on 0.2% glucose-salts medium was inoculated into 500 ml of fresh salts medium and grown with aeration on 0.2% glucose from E_{420} 0.1 to 1.0. The cells were then cooled on ice, harvested by centrifugation, washed in 0.01 M-potassium phosphate buffer, pH 7.0, and resuspended in 2 litres of fresh salts medium to give E_{420} 0.2-0.3. The cells were grown in a medium with limiting glucose (0.04%, w/v), harvested just at the completion of growth, washed in 200 ml of phosphate buffer, and resuspended in buffer to a protein concentration of 6.25 mg/ml. The suspension was then aerated at 37°C for 1-1½ h to ensure the depletion of substrate as well as to oxidize the electron transport system before determination of difference spectra. To prepare sonic extracts, the cells were suspended in 0.1 M-potassium phosphate buffer, pH 8.0 (1 g

wet wt. of cells/15ml of buffer), and then disrupted by treatment in a Bronwill 20kHz ultrasonic oscillator for 4 min.

For anaerobic cells, the cultures were first grown aerobically from E_{420} 0.1 to 0.35 in 1 litre of salts medium containing 650 μ g of glucose/ml, then transferred into a 2-litre Erlenmeyer flask fitted with a two-hole stopper and N_2 gas was bubbled through an inlet tube vigorously for 10 min. A glass exit tube, placed 1.3 cm above the culture, allowed gases to escape. With anaerobic conditions being maintained, the cells were grown at 37°C until the glucose was exhausted ($E_{420} \approx 1.0$). Portions (4 ml) were removed at intervals throughout the aerobic and anaerobic stages for both protein and glucose determinations. At the end of growth the 37°C bath was replaced by an ice-salt bath while N_2 flow was continued for 1 h. The cells were then harvested, washed in 0.01 M-potassium phosphate buffer, pH 7.0, and the difference spectrum was studied immediately.

(b) Growth of cells for enzyme assay. Cultures were grown for 18 h, centrifuged and resuspended in fresh salts medium supplemented with 0.25 mM-2,4-dinitrophenol and 0.2% glucose. The culture was grown from E_{600} 0.06 to 0.5. The cells were harvested, washed once in 0.01 M-potassium phosphate buffer, pH 7.0, and then resuspended in 500 ml of salts medium containing 0.2% glucose in each of two flasks, designated as the control flask without 2,4-dinitrophenol, and the 2,4-dinitrophenol flask (0.25 mM-2,4-dinitrophenol). Both cultures were grown with aeration at 37°C until they reached E_{600} 0.47 at which point they were chilled in ice, harvested by centrifugation, washed in 0.01 M-potassium phosphate buffer, pH 8.0, and stored at 0°C as packed cells.

To obtain sonic extracts, the cells were resuspended in 0.1 M-potassium phosphate buffer, pH 8.0, to a final concentration of 1 g wet wt. of cells/15 ml of buffer. A 30 s treatment of the cells in a Branson model W 1350 Sonifier effected disruption.

Determination of difference spectra. Cell suspensions were placed in cuvettes in both the upper and lower compartments of the Cary 15 spectrophotometer. Substrate (0.2% glucose, gluconate or succinate) was then added to the upper cuvette to produce a reduced-oxidized difference spectrum.

Chemical analyses. Glucose was determined by using the commercial preparation Glucostat (Worthington Biochemical Corp., Freehold, N.J., U.S.A.) as described by Coukell & Polglase (1969).

Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine γ -globulin (California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A.) as standard.

Enzyme assays. All enzyme activities were determined on freshly prepared crude sonic extracts by using the Cary 15 spectrophotometer at 25°C. Fumarase (EC 4.2.1.2) activity and aconitase (EC 4.2.1.3) activity were determined at 240 nm by following the method of Hanson & Cox (1967). A unit of activity for both enzymes is defined as ΔE_{240} 0.001/min.

Glucose 6-phosphate dehydrogenase (EC 1.1.1.49) was determined by measuring the reduction rate of $NADP^+$ by glucose 6-phosphate at 340 nm. The reaction mixture (1 ml) contained: glucose 6-phosphate, 5 μ mol; $MgCl_2$, 10 μ mol; $NADP^+$, 0.42 μ mol; glycylglycine buffer, pH 7.5,

50 μ mol; crude extract (0.05 mg of protein). A unit of activity is defined as that amount of enzyme effecting the formation of 1 nmol of NADPH/min.

Specific activities were calculated as units of enzyme/mg of protein. The values for enzyme activities are averages from several determinations.

RESULTS

Yield of cell protein from glucose for *E. coli* B and the streptomycin-dependent mutant. It was reported previously (Coukell & Polglase, 1969) that the weight of cells produced from on a limiting-glucose medium was about 35% less for streptomycin-dependent *E. coli* B than for the wild-type strain when cultures were grown aerobically. A proportional difference was observed when the yield of protein was determined for each strain. However, on changing to a nitrogen atmosphere, both strains produced equivalent quantities of cell protein, indicating that the metabolic deficiency of streptomycin-dependent *E. coli* B was in an aerobic energy-yielding process (Fig. 1). The yield of protein on a limiting-glucose medium, for the non-dependent revertant strain (B-R₄) grown aerobically, corresponded to that of the streptomycin-dependent strain from which it was derived.

Difference spectra of wild-type and streptomycin-dependent *E. coli* B. Wild-type *E. coli* B possessed the 'a' cytochromes (600–650 nm), cytochrome *b* (560 nm), the flavin trough (about 460 nm) and a large, symmetrical peak at 503 nm (Fig. 2a). The

Fig. 1. Yield of cell protein from glucose for *E. coli* B (■) and the streptomycin-dependent mutant (□). The initial concentration of glucose used was 650 μ g/ml. After a preliminary period of aerobic growth, the atmosphere was changed (at the arrows) to N_2 gas.

Fig. 2. (a) Reduced-oxidized difference spectra after addition of glucose to air-oxidized cell suspensions. —, Wild-type *E. coli* B; ---, streptomycin-dependent *E. coli* B;, non-dependent revertant *E. coli* B (R₄). (b) Reduced-oxidized difference spectra after addition of glucose to air-oxidized cell suspensions. ---, Wild-type *E. coli* K-12;, streptomycin-dependent *E. coli* K-12.

streptomycin-dependent mutant and the revertant strain derived from the dependent mutant gave difference spectra that lacked the 503nm pigment (Fig. 2a). Similarly wild-type *E. coli* K12 possessed the 503nm pigment, whereas the peak failed to appear in the streptomycin-dependent mutant of this strain (Fig. 2b). These results suggested that the lack of the 503nm pigment and the impaired energy metabolism observed in streptomycin-

Fig. 3. Growth with limiting glucose of wild-type *E. coli* B on minimal salts medium supplemented with 2,4-dinitrophenol: —, none; ---, 0.1 mM; . . ., 0.25 mM; - · - · -, 0.5 mM.

dependent strains of *E. coli* (and the non-dependent revertant) were related phenomena.

Effect of 2,4-dinitrophenol on the 503nm pigment in wild-type cells. When *E. coli* B (wild-type) was grown on a limiting-glucose medium on various concentrations of 2,4-dinitrophenol (Fig. 3) it was found that at 0.25 mM-2,4-dinitrophenol the growth rate and yield of cell protein were similar to that of streptomycin-dependent *E. coli* B (Fig. 3). Growth of *E. coli* B with 10 μ M-2,4-dinitrophenol on a limiting-glucose medium decreased the height of the 503nm peak by half and growth with 0.1 mM eliminated it completely (Fig. 4). Experiments in which cells grown without added 2,4-dinitrophenol were subsequently treated with 2,4-dinitrophenol, indicated that 2,4-dinitrophenol had no effect on reduction of the 503nm pigment by glucose. It was possible, then, by growing cells with 0.1–0.25 mM-2,4-dinitrophenol, to eliminate the 503nm pigment and to decrease the aerobic yield of cell protein.

Effect of 2,4-dinitrophenol on enzyme activities. De-repression of catabolite-repressible enzymes was observed in streptomycin-dependent *E. coli* (Coukell & Polglase, 1969) and a similar effect would be expected with wild-type *E. coli* grown with

Fig. 4. Reduced-oxidized difference spectra after addition of glucose to air-oxidized suspensions of wild-type *E. coli* B grown in medium supplemented with 2,4-dinitrophenol: —, none; ----, 10 μ M;, 0.1 mM.

2,4-dinitrophenol. When *E. coli* B was grown with excess of glucose in a medium containing 0.25 mM-2,4-dinitrophenol, fumarase showed a sixfold increase and aconitase a twofold increase in specific activity (Table 1).

An unexpected observation was the de-repression in 2,4-dinitrophenol-grown wild-type cells of glucose 6-phosphate dehydrogenase, an enzyme not known to be sensitive to catabolite repression. Although the specific co-repressor for glucose 6-phosphate dehydrogenase is not known one might speculate that de-repression of this enzyme could result from rapid utilization of its product, NADPH, which may transfer its hydrogen to the 2,4-dinitrophenol in the medium. (The 2,4-dinitrophenol-containing medium undergoes a change in colour when wild-type *E. coli* B cells are grown in it.) It is conceivable that the 2,4-dinitrophenol substitutes for the 503 nm pigment as a hydrogen acceptor from NADPH. This speculation implies that NADPH may also serve as an inducer for the 503 nm pigment in *E. coli* cells growing normally.

Difference spectra with gluconate and succinate. Further evidence for the production of the 503 nm band by hydrogen transfer from NADPH is shown in Fig. 5(a). When gluconate (for which NADP⁺ is the first electron acceptor) was added to oxidized *E. coli* B cells, only the 503 nm pigment was prominent in the first 1–2 min. Subsequently, the cytochromes became reduced. In contrast, when succinate (for which flavin is the first electron acceptor) was added to oxidized *E. coli* B cells (Fig. 5b) the 503 nm component failed to appear.

Table 1. Specific activities of enzymes from *E. coli* B. grown on glucose with or without 2,4-dinitrophenol

The minimal salts medium contained glucose at an initial concentration of 0.2%. Enzymes were assayed in ultrasonically-treated extracts of cells harvested at E_{600} 0.47. Specific activities are mean values obtained from six experiments.

Supplement ... Enzyme	Specific activity (units/mg of protein)	
	None	2,4-Dinitrophenol (0.25 mM)
Fumarase	400	2400
Aconitase	200	360
Glucose 6-phosphate dehydrogenase	110	450

Effect of anaerobic growth on the 503 nm pigment. The report by Nosoh (1964), that the 503 nm pigment is present when wild-type cells are grown anaerobically, was verified. In fact, this component, and cytochrome *b*, were in an oxidized state after anaerobic growth, indicating that they were not functional in the absence of oxygen.

Effect of sonic treatment on difference spectra. Lindenmayer & Smith (1964) noted the disappearance of the 503 nm peak on disruption of yeast cells. Likewise, when the conditions used for generating the spectrum of the 503 nm pigment in *E. coli* B whole cells were applied to extracts prepared by sonic disruption, the pigment failed to appear.

DISCUSSION

The results reported here provide indirect evidence that the 503 nm pigment plays a role in aerobic energy metabolism. Streptomycin-dependent *E. coli* (and a non-dependent revertant) are deficient in energy metabolism and a similar deficiency was observed in wild-type cells grown in medium containing 2,4-dinitrophenol. There appeared to be a correlation between impaired energy metabolism and the lack of the 503 nm pigment. Catabolite repression of fumarase and aconitase was relaxed in 2,4-dinitrophenol-grown wild-type cells as well as in streptomycin-dependent *E. coli* (Coukell & Polglase, 1969) presumably as a result of impaired energy production. With regard to the foregoing properties, 2,4-dinitrophenol-grown *E. coli* B and streptomycin-dependent *E. coli* B were similar. However, 2,4-dinitrophenol-grown wild-type cells showed, in addition, a fourfold increase in glucose 6-phosphate dehydrogenase activity, a property not observed in streptomycin-dependent *E. coli*. This observation suggested that, in wild-

Fig. 5. (a) Reduced-oxidized difference spectra after addition of gluconate to an air-oxidized suspension of wild-type *E. coli* B: . . . , after 2 min; —, after 5.2 min. (b) Reduced-oxidized difference spectra after addition of glucose (—) or succinate (----) to air-oxidized suspensions of wild-type *E. coli* B.

type cells growing in the presence of 2,4-dinitrophenol, NADPH (a product of glucose 6-phosphate dehydrogenase activity) was being oxidized more

rapidly than in non-treated cultures, possibly by electron transfer to 2,4-dinitrophenol. This led to the speculation that the normal role of the 503nm pigment is that of an electron acceptor from NADPH.

The effect of gluconate (for which NADP^+ is a hydrogen acceptor) on the difference spectrum provided further evidence for the involvement of NADPH, since with gluconate as substrate the 503nm pigment was prominent in wild-type cells before the appearance of reduced cytochromes. Further, the addition of succinate (for which flavin is the electron acceptor) to oxidized *E. coli* cells failed to generate a peak at 503nm, even after prolonged incubation, although reduced cytochrome bands were formed rapidly with this substrate.

On the basis of these results it may be postulated that the 503nm pigment of *E. coli* is a constituent of an oxidative energy-yielding pathway for which NADPH is the initial substrate. Any hypothesis advanced on the basis of the indirect experimental evidence reported here must be regarded as speculative. However, such speculation may be justified by the lack of information on the role of the 503nm pigment, the occurrence of which has been known for several years.

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