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Nitrate Reductase Complex of *Escherichia coli* K-12: Participation of Specific Formate Dehydrogenase and Cytochrome b_1 Components in Nitrate Reduction

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The participation of distinct formate dehydrogenases and cytochrome components in nitrate reduction by *Escherichia coli* was studied. The formate dehydrogenase activity present in extracts prepared from nitrate-induced cells of strain HfrH was active with various electron acceptors, including methylene blue, phenazine methosulfate, and benzyl viologen. Certain mutants which are unable to reduce nitrate had low or undetectable levels of formate dehydrogenase activity assayed with methylene blue or phenazine methosulfate as electron acceptor. Of nine such mutants, five produced gas when grown anaerobically without nitrate and possessed a benzyl viologen-linked formate dehydrogenase activity, suggesting that distinct formate dehydrogenases participate in the nitrate reductase and formic hydrogenlyase systems. The other four mutants formed little gas when grown anaerobically in the absence of nitrate and lacked the benzyl viologen-linked formate dehydrogenase as well as the methylene blue or phenazine methosulfate-linked activity. The cytochrome b_1 present in nitrate-induced cells was distinguished by its spectral properties and its genetic control from the major cytochrome b_1 components of aerobic cells and of cells grown anaerobically in the absence of nitrate. The nitrate-specific cytochrome b_1 was completely and rapidly reduced by 1 mM formate but was not reduced by 1 mM reduced nicotinamide adenine dinucleotide; ascorbate reduced only part of the cytochrome b_1 which was reduced by formate. When nitrate was added, the formate-reduced cytochrome b_1 was oxidized with biphasic kinetics, but the ascorbate-reduced cytochrome b_1 was oxidized with monophasic kinetics. The inhibitory effects of *n*-heptyl hydroxyquinoline-*N*-oxide on the oxidation of cytochrome b_1 by nitrate provided evidence that the nitrate-specific cytochrome is composed of two components which have different redox potentials but identical spectral properties. We conclude from these studies that nitrate reduction in *E. coli* is mediated by the sequential operation of a specific formate dehydrogenase, two specific cytochrome b_1 components, and nitrate reductase.

A number of observations indicate that nitrate reduction in *Escherichia coli* occurs mainly by a pathway involving formate dehydrogenase, cytochrome b_1, and nitrate reductase. Among the various substrates which are metabolized by *E. coli*, formate is the most effective electron donor for the reduction of nitrate in cells grown anaerobically in the presence of nitrate (2, 23). In such cells, formate dehydrogenase, cytochrome b_1, and nitrate reductase are induced to relatively high levels compared to those in cells grown in the absence of nitrate (2, 17, 23). These three components are present in membrane fractions (6) and have been partially purified as a unit from cell extracts (8). Finally, mutants of *E. coli* which are unable to produce nitrite from nitrate (NR^- mutants) lack either formate dehydrogenase or nitrate reductase or combinations of these activities and cytochrome b_1 (1, 16, 17, 22).

The proposal that formate dehydrogenase and cytochrome b_1 are specific components of the nitrate reduction system raises some important

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questions concerning the relationship of this pathway with other pathways of electron transport in *E. coli*. Formate dehydrogenase must also be a component of the hydrogenlyase system (15) as well as of formate oxidase. These multiple functions of formate dehydrogenase have been previously recognized, and it has been suggested that at least two distinct formate dehydrogenases, particulate and soluble, are formed by *E. coli* (4, 5).

The participation of cytochrome b_1 in nitrate reduction creates an even more complex situation, since cytochrome b_1 is one of the major cytochromes present in both aerobic and anaerobic cells of *E. coli* and therefore must function in other electron transport systems (3, 10, 18). At present it is not known whether distinct cytochrome b_1 components are involved in different electron pathways or to what degree such pathways interact.

To define the components of the nitrate reductase complex and their relationship to other metabolic pathways, we studied the biochemical events involved in nitrate reduction in the wild-type *E. coli* and in a number of NR^- mutants. Evidence is presented that a specific formate dehydrogenase, two distinct cytochrome b_1 components, and nitrate reductase are obligatory components of a nitrate reductase complex in *E. coli*.

MATERIALS AND METHODS

The strains of *E. coli* used in these studies, HfrH and AB2102, and the NR^- mutants derived from them were described previously (17). All strains were grown on a complete medium containing a salt base (20), thiamine hydrochloride (5 μg/ml), 0.4% nutrient broth (Difco), and 1% glucose (sterilized separately). When indicated, the medium was supplemented with 1% potassium nitrate and 0.5% sodium formate.

For measuring activities, cultures were grown in flasks with Klett tube side arms at 37 C in a shaking water bath. For aerobic conditions, the medium was sparged vigorously with sterile air. For anaerobic conditions, the medium was sparged with a sterile mixture of 95% N_2 plus 5% CO_2. The cultures were inoculated with a 2 to 5% inoculum from an overnight culture grown in similar medium and allowed to grow to the middle of the exponential phase. The cells were collected by centrifugation, washed twice with 0.05 M potassium phosphate buffer (*p*H 6.8 or 7.3), and resuspended in the same buffer. This suspension was used as such (whole cells) or frozen overnight at −15 C before use (frozen-thawed cells). Cell-free extracts were prepared by three treatments of 15 sec each in a 10-kc Branson Sonifier, followed by centrifugation at 3,000 × *g* for 10 min to remove whole cells.

Formate dehydrogenase was measured with different electron acceptors by utilizing a radioassay or a spectrophotometric assay described previously (17), or by the following manometric procedure using a Warburg apparatus. In the main compartment were placed 0.2 ml of cells or extract, 10 μmoles of electron acceptor, and 0.05/M potassium phosphate buffer (*p*H 6.8) in a total volume of 2.7 ml; 30 μmoles of formate was placed in the side arm. Flasks and manometers were flushed with N_2 and equilibrated at 37 C before tipping the formate into the main compartment. Activity was expressed as microliters of CO_2 evolved per minute per milligram of protein. No CO_2 was produced in the absence of added electron acceptors.

In some experiments, benzyl viologen-specific formate dehydrogenase was measured by the following qualitative procedure. In Thunberg tubes, 140 μmoles of potassium phosphate buffer (*p*H 6.8), 1.0 μmoles of benzyl viologen, 60 μmoles of sodium formate, and cells or extract were mixed in a final volume of 5.0 ml. The tubes were flushed with N_2, and reduction of the benzyl viologen was followed by use of a Klett colorimeter with a 660-nm filter. The development of color was not linear and, therefore, the results were expressed qualitatively.

Formic hydrogenlyase was assayed manometrically by following hydrogen evolution from formate (15). Gas production by growing cultures was assessed by observing the accumulation of gas in inverted vials submerged in tubes of the culture medium.

Nitrate reductase was assayed with formate or reduced methyl viologen as electron donors, as previously described (17). With other electron donors the procedure utilized with formate was followed, substituting the electron donor for formate.

The absorption spectra of the cytochromes were determined with a single-beam recording spectrophotometer constructed and kindly made available by Warren Butler at the University of California, San Diego. Monochromatic light of 1.0-nm half bandwidth was provided by a Bausch & Lomb grating monochromator. The output of the photometer and a potentiometer geared to the wavelength drum provided the input signals to a Moseley 7000 AM X-Y recorder. For measurement of absolute reduced spectra at liquid nitrogen temperature, a 1.0-ml sample was placed in a metal-sided absorption cell, reduced with solid sodium dithionite, mixed with 500 mg of Al_2O_3 (1 μm diameter), frozen in liquid N_2, and placed in a Dewar flask containing N_2 in the cell compartment of the instrument. For measurement of absolute reduced spectra at room temperature, the same general procedure was followed, omitting the liquid N_2. For determining the differential spectra, an attachment which provided a split beam was utilized. In this case, identical 3.0-ml samples were placed in glass absorption cells (10-nm light path). The contents of one cuvette was oxidized by flushing with air for 30 sec, and the other was reduced with solid sodium dithionite to obtain the reduced-minus-oxidized spectrum.

Cytochrome levels were calculated as follows. A basal line was drawn between 540 and 570 nm, and the height of the peak of the α-band was measured from this baseline. The optical density units were calcu-

lated, and the cytochrome level is expressed as optical density units per milligram of protein. The kinetics of reduction and oxidation of cytochrome b_1 was followed with an Aminco-Chance double-wavelength spectrophotometer at either 35 C or room temperature. Protein was determined by the procedure of Lowry et al. (12).

RESULTS

The general properties of the nitrate reduction system present in the wild-type strains utilized in these studies corresponded to those previously reported (2, 6, 8). In nitrate-induced cells of strains HfrH, formate was a more effective electron donor for nitrate reduction than glucose (Table 1). In frozen-thawed cells or in cell extracts, several reagents act as electron donors for nitrate reduction (Table 1). Reduced methyl viologen, which transfers electrons directly to nitrate reductase (21), was the most effective electron donor, but formate was 30 to 40% as active as reduced methyl viologen in frozen-thawed cells. Nitrate reduction with formate as electron donor was completely inhibited by 0.01 mM *n*-heptyl hydroxyquinoline-*N*-oxide (HOQNO), whereas a 100-fold increase in the concentration of the inhibitor did not affect the reduction of nitrate with reduced methyl viologen or ascorbate as the electron donor.

The formate dehydrogenase present in nitrate-induced cells of strain HfrH utilized several electron acceptors (Table 2). Methylene blue and phenazine methosulfate were the most effective acceptors, whereas potassium ferricyanide, benzyl viologen, and triphenyltetrazolium chloride were much less effective. We have routinely measured formate dehydrogenase by following the reduction of dichlorophenol indophenol (DCPIP) in the presence of catalytic amounts of phenazine methosulfate (17). Although DCPIP is only slowly reduced by a mixture of crude extract and formate, in the presence of small amounts of phenazine methosulfate it is reduced at a rate which corresponds to the rate observed with methylene blue. This assay does not involve an indirect flow of electrons through cytochrome b_1, since reduced cytochrome b_1 is not oxidized by phenazine methosulfate in our preparations and formate dehydrogenase can be assayed by this procedure in mutants which lack cytochrome b_1.

The availability of a number of mutants which lack formate dehydrogenase in nitrate-induced cells (17) permitted us to test directly the possibility that distinct formate dehydrogenases are involved in the metabolism of *E. coli*. Although formate dehydrogenase activity which utilizes methylene blue or phenazine methosulfate as electron acceptors could not be detected or was very low in such mutants, some of the mutants still formed gas when cultivated under conditions which lead to the formation of the formic hydrogenlyase system in the wild-type strain (Table 3). When benzyl viologen was used as an electron

TABLE 1. *Nitrate reduction with various electron donors*[a]

Electron donor	Nitrate reduction activity[b]		
	Whole cells	Sonically disrupted cells	Frozen-thawed cells
(A) Glucose	0.14		
Formate	2.21	0.15	1.86
Methyl viologen (reduced)		4.36	3.46
(B) Methyl viologen (reduced)			1.18
Dithionite			0.21
Ascorbate			0.02
Formate			0.72

[a] Nitrate-induced cells were grown and treated as described. Nitrate reduction was assayed as described, with the following levels of electron donors in a final volume of 2.5 ml: 48 μmoles of glucose, 48 μmoles of formate, 0.25 μmoles of methyl viologen (reduced), 2.8 μmoles of sodium dithionite, 10 μmoles of ascorbate, 6 μmoles of formate.

[b] Expressed as micromoles of nitrate per minute per milligram of protein.

TABLE 2. *Activity of formate dehydrogenase with various electron acceptors*

Electron acceptor[a]	Activity[b]
None	0.6
Methylene blue	65.2
Phenazine methosulfate + DCPIP	56.7
DCPIP	15.8
Ferricyanide	3.5
Benzyl viologen	12.2
Triphenyl tetrazolium chloride	9.0
Air	12.3

[a] The concentration of electron acceptors used was 3.1 μmoles/ml (except for air).

[b] Nitrate-induced cells of HfrH were grown and broken in a Branson Sonifier, and the extract was prepared as indicted in Materials and Methods, but it was frozen before use. Formate dehydrogenase was assayed by the release of radioactive CO_2 from ^{14}C-formate, and nanomoles of CO_2 were calculated from the specific activity of the formate (8,230 counts per min per μmole) utilized. Results shown are expressed as nanomoles of CO_2 per minute per milligram of protein.

acceptor, the evolution of CO_2 from formate or the reduction of the dye in the presence of formate was not linear with time and is, therefore, expressed qualitatively.

These results support the hypothesis that two biochemically distinct formate dehydrogenases are involved in nitrate reduction and hydrogen formation in *E. coli*. However, the formate dehydrogenases may not be genetically distinct since, in some of the NR^- mutants, both the nitrate-specific formate dehydrogenase and the formate dehydrogenase involved in the formic hydrogenlyase system are lost (Table 3) as the result of mutations which appear to be single, point mutations on the basis of reversion rates (17).

When *E. coli* is grown anaerobically, the addition of nitrate causes an increase in the level of cytochrome b_1 (17, 23). The following spectral analysis demonstrates that the cytochrome b_1 components formed in the presence and absence of nitrate are also qualitatively distinct. Absolute spectra of frozen-thawed cells reduced with dithionite are shown in Fig. 1. The cells grown in the presence of nitrate exhibited only two major peaks between 500 and 600 nm. These were 526 nm (β band) and 555 nm (α band) at liquid nitrogen temperatures and 528 and 558 nm at room temperature. On the other hand, the cells grown in the absence of nitrate exhibited major peaks at 528 and 558 nm, a shoulder at 549 nm (cytochrome *c*) at liquid nitrogen temperature, and peaks at 530 and 560 nm at room temperature. The distinction between these b_{555} and b_{558} components (identified by their α band peaks at liquid nitrogen temperature) was clear in some NR^- mutants which had reduced levels of cytochrome b_1. When such mutants were grown under anaerobic conditions in the presence of nitrate, both components were apparent in the reduced spectra (Fig. 2, mutant TW-15). Furthermore, a second type of mutant, which formed no detectable cytochrome b_{555} component when grown anaerobically with nitrate (Fig. 2, strain RB-12), formed normal levels of the b_{558} components when grown anaerobically in the absence of nitrate (not shown, cf. wild-type-uninduced spectrum, Fig. 1).

When the wild-type strains were grown aerobically, the bands of the major cytochrome *b* components were at 555 and 562 nm (at liquid nitrogen temperature). On the basis of the following observations with NR^- mutants, the b_{555} components found in anaerobic, nitrate-induced cells would appear to be distinct from the b_{555} component in aerobic cells. NR^- mutants, which have altered levels of the b_{555} component when grown anaerobically with nitrate, formed a normal distribution of cytochrome components, including a b_{555} component, when they were grown under aerobic conditions (Fig. 2). The absolute spectra shown for the aerobic cells are identical to that found in aerobic wild-type cells grown under these conditions, showing peaks at 555 and 562 nm.

Thus the major cytochrome *b* component found in cells grown anaerobically in the presence of nitrate appears to be physiologically and genetically distinct from the cytochrome *b* components found under other growth conditions. The following observations directly implicate the b_{555} component in the nitrate reduction system and provide evidence that it is composed of two distinct cytochromes which possess identical spectral characteristics.

TABLE 3. *Gas formation and formate dehydrogenase activity in HfrH and selected NR^- mutants*

Strain	Gas formation[a]	Formate dehydrogenase activity[b]		
		Methylene blue[c]	Phenazine methosulfate + DCPIP	Benzyl viologen
HfrH	+	1.15	0.43	+
TW-15	+	0.05	0.02	+
TW-17	+	0.08	0.02	+
TW-22	0	0.00	0.00	0
TW-101	+	0.09	0.02	+
TW-112	+	0.04	0.00	+
TW-135	0	0.00	0.00	0
TW-140	0	0.00	0.00	0
TW-149	0	0.14	0.00	0
TW-153	+	0.00	0.00	+

[a] Gas formation was assessed in cultures growing anaerobically in complete medium without nitrate in tubes containing inverted vials to trap gas. Cultures were allowed to incubate at least 5 days and were considered to be positive (+) if they formed more than 10% the gas observed in the wild type.

[b] The assay of formate dehydrogenase with benzyl viologen was qualitative. All assays were performed with fresh cell extracts prepared from cells grown anaerobically on complete medium (benzyl viologen assay) or on complete medium supplemented with nitrate (radioassay and colorimetric assay). Results are expressed as micromoles of CO_2 per minute per milligram of protein; results for benzyl viologen are expressed qualitatively.

[c] The activity with methylene blue was calculated as micromoles of CO_2 released from formate (specific activity, 22,800 counts per min per μmole).

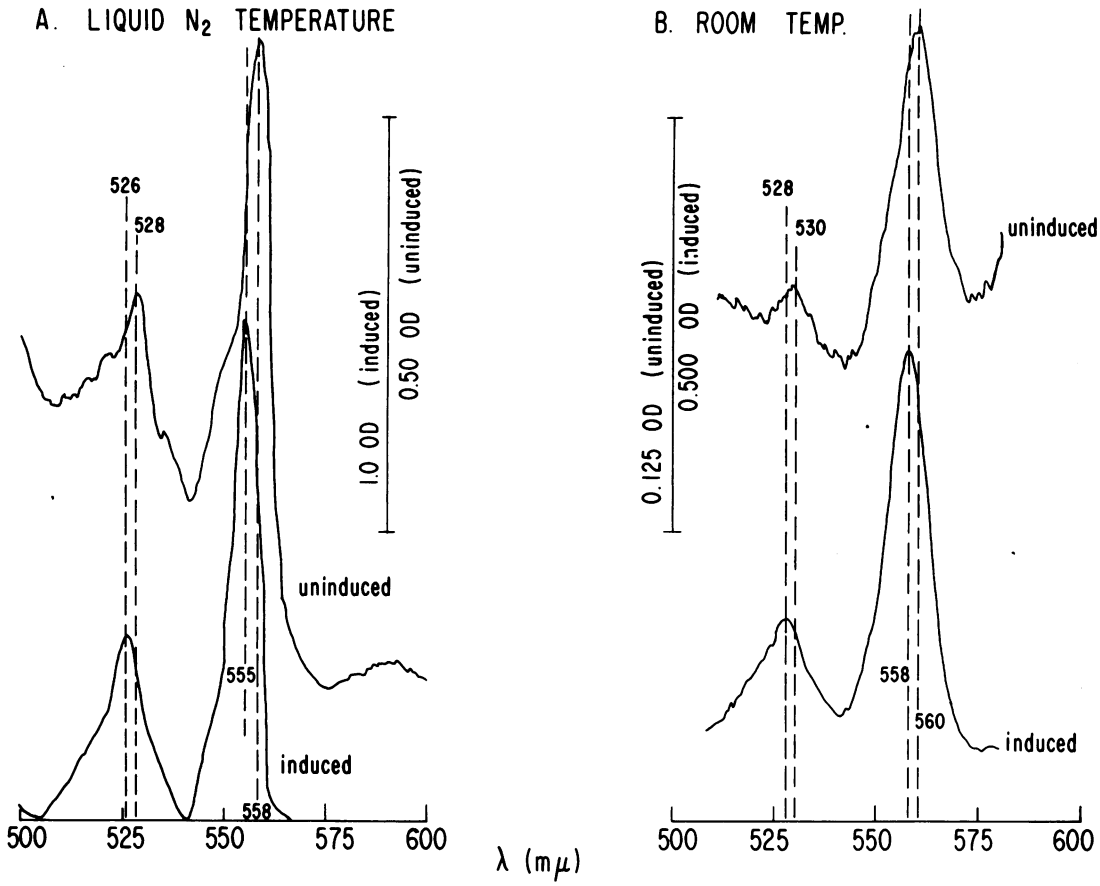

FIG. 1. *Absolute dithionite-reduced spectra of strain HfrH grown with and without nitrate. Cells were grown anaerobically in complete medium with (induced cells) and without (uninduced cells) potassium nitrate and treated as indicated. Frozen-thawed cell suspensions were used at protein concentration of 3.08 mg/ml for the uninduced cells and 1.74 mg/ml for the induced cells. Spectra were determined at liquid N_2 temperature (A) and room temperature (B). The bars in the center of the figure show the scale of optical density units for each of the curves.*

When the kinetics of reduction and oxidation of cytochrome b_1 were followed in an Aminco-Chance double-wavelength recording spectrophotometer, it was found that repeated additions of 1 mM reduced nicotinamide adenine dinucleotide (NADH) did not cause the reduction of the cytochrome b_1 component present in frozen-thawed preparations of nitrate-induced cells (Fig. 3). By increasing the NADH concentration to 100 mM, it was possible to effect a reduction of cytochrome b_1, but we regarded this as a non-physiological level of NADH. Similar results were obtained by using crude extracts. When 1 mM formate was added, an immediate reduction of cytochrome b_1 occurred. The subsequent addition of nitrate caused the complete oxidation of the cytochrome, but with peculiar kinetics (Fig. 3). Only part of the cytochrome was immediately oxidized, the remainder being oxidized after a definite time lag. With some time lag, ascorbate also caused the reduction of cytochrome b_1. However, in this case the reduced cytochrome was completely oxidized in a single step upon addition of nitrate (Fig. 4A). Apparently only part of the cytochrome b_1 was reduced, since the subsequent addition of formate caused the reduction of more cytochrome, and in this case the biphasic kinetics were observed for oxidation by nitrate. This difference in the extent of reduction of the cytochrome component caused by formate and ascorbate was verified by the experiment shown in Fig. 4B, in which the addition of formate immediately after ascorbate caused an additional increment of cytochrome reduction. These results suggested that two cytochrome b_1 components were reduced by formate, one of which is reduced by ascorbate, and that nitrate oxidized both cytochrome components but one of them only after a definite time lag.

The effects of HOQNO on the oxidation of

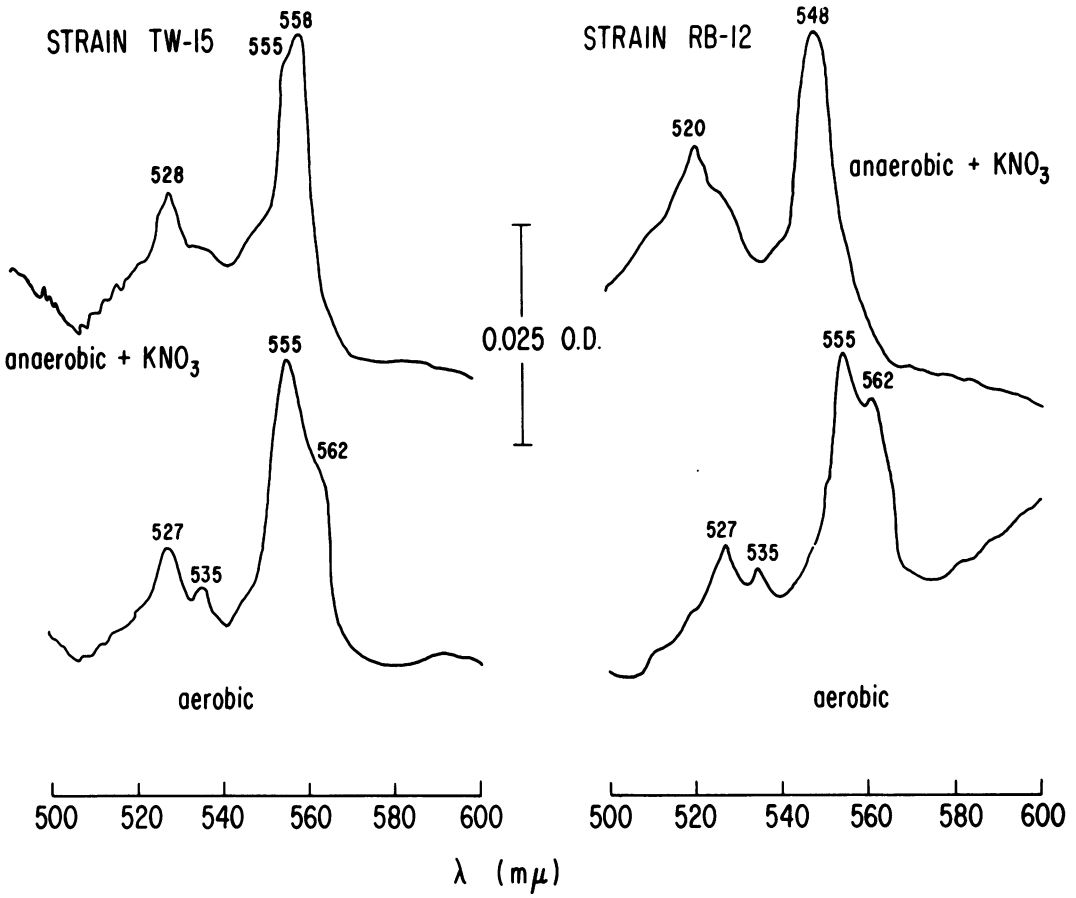

FIG. 2. *Cytochrome spectra of two NR⁻ mutants grown under different conditions. The spectra were determined at liquid N_2 temperature on frozen-thawed suspensions of cells grown and prepared as described. Protein concentrations were 6.25 mg/ml for RB-12 (anaerobic), 4.32 mg/ml for RB-12 (aerobic), 6.82 mg/ml for TW-15 (anaerobic), and 5.72 mg/ml for TW-15 (aerobic).*

cytochrome b_1 component provided additional evidence that two distinguishable cytochromes were being reduced and oxidized in these experiments. When 0.01 mM HOQNO was added after the complete reduction of the cytochrome (Fig. 4B), only part of the cytochrome was reoxidized by nitrate and the oxidation took place immediately in a single step. Identical results were obtained when the cytochrome was reduced by formate only, i.e., the second step of the biphasic kinetics was completely inhibited by HOQNO. Furthermore, the reduction again of the oxidized cytochrome by formate was prevented by HOQNO (Fig. 4B). In contrast, that portion of cytochrome b_1 which was reduced by ascorbate was not prevented by HOQNO from being oxidized by nitrate (Fig. 4C). The addition of more nitrate had no further effect and, in this case, HOQNO did not prevent formate from reducing at least part of the cytochrome.

To demonstrate that these results were due specifically to the behavior of cytochrome b_{555}, a similar set of experiments was carried out in which spectra were determined at room temperature with the split-beam attachment for the spectrophotometer (Fig. 5A). The formate-reduced cytochrome *b* exhibited an α band, at 558 nm at room temperature, which corresponds to the b_{555} component (*see* Fig. 1B). Nitrate completely reoxidized the cytochrome, but when HOQNO was added to the formate-reduced preparation, nitrate caused the oxidation of only part of the cytochrome, leaving a reduced cytochrome with an identical absorption maximum. The results presented in Fig. 5B also confirmed the results obtained with ascorbate as electron donor and demonstrated that a cyto-

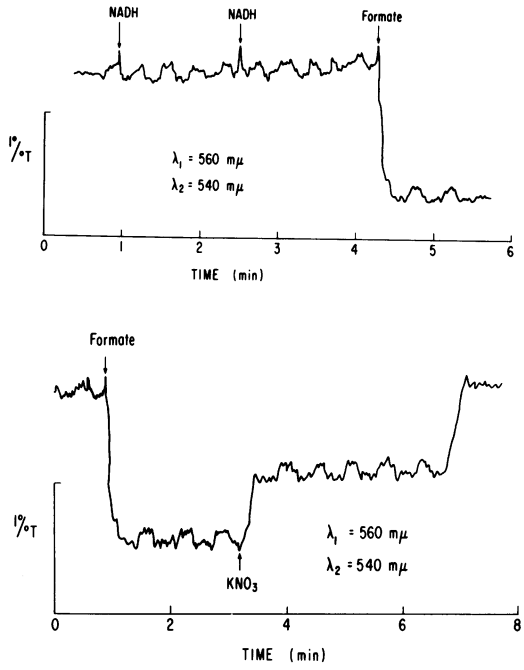

FIG. 3. *Reduction of cytochrome b_1 and its reoxidation by nitrate. Changes in the state of the cytochrome were followed as the difference in transmittance between λ_1 (560 nm) and λ_2 (540 nm) at 35 C. The scale at the left shows the scale of transmittance change observed. Cells of HfrH were grown anaerobically in complete medium with 1% potassium nitrate, washed, and resuspended as described. A frozen-thawed suspension (1.74 mg of protein) was mixed with 0.05 M potassium phosphate buffer, pH 6.8, in a final volume of 2.8 ml. Where indicated, 3.8 μmoles of NADH (0.01 ml), 3.8 μmoles of sodium formate (0.01 ml), and 10 μmoles of potassium nitrate (0.01 ml) were added.*

chrome with an absorption maximum of 558 nm at room temperature is involved. The kinetic changes observed, therefore, reflect changes occurring specifically in the cytochrome b_{555} components.

These results can be explained assuming the participation in nitrate reduction of two cytochromes with identical spectra differing only in their redox potentials (*see* Fig. 7).

Further evidence for such a hypothesis was obtained by analyzing the autooxidation of cytochrome b_1 in nitrate-induced cells (Fig. 6). Formate at a concentration of 8.3×10^{-3} M rapidly reduced cytochrome b_1 (curve a). After air was bubbled through the cell suspension for 1 min, the cytochrome was completely oxidized (curve b). When 8.3×10^{-5} M HOQNO was added, bubbling with air for 1 min caused rapid oxidation of only half of the cytochrome, and the remainder of the reduced cytochrome was not oxidized, even after 8 min of continued bubbling with air (curve c). When the concentration of formate was lower (5×10^{-4} M), HOQNO did not inhibit the oxidation of the cytochrome (curves d, e, f).

Again, these results may be interpreted by assuming the existence of two cytochromes in nitrate-induced cells which can be reduced by formate and oxidized by oxygen (Fig. 7). The presence of HOQNO would inhibit the electron flow between both cytochromes, allowing only one cytochrome to be rapidly oxidized in the presence of excess formate, since formate would continue to reduce the cytochrome before the block. The fact that HOQNO had no effect on oxidation of cytochromes when a low level of formate was used suggests that both cytochromes are autooxidizable.

DISCUSSION

The participation of formate dehydrogenase, cytochrome b_1 and nitrate reductase in nitrate

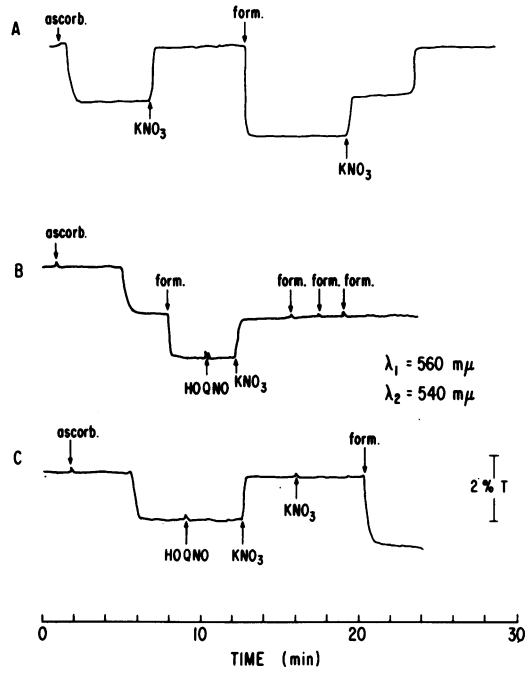

FIG. 4. *Reduction and oxidation of cytochrome b_1 and effect of HOQNO. Cells were prepared and the conditions were as in Fig. 3 except that the frozen-thawed cell suspension was treated with deoxyribonuclease (2.5 μg/ml) to reduce the viscosity. Sodium formate and sodium ascorbate (ascorbic acid adjusted to pH 7.0 with sodium hydroxide) were added as 0.01 ml of 1.0 M solutions to 4.0 ml of cell suspension (4.8 mg/ml).*

reduction by *E. coli* has been proposed by a number of investigators (6, 8, 17, 23). The results presented here, along with the results from studies on NR$^-$ mutants (17), indicate that distinct forms of these components are involved in the nitrate reduction system and that there is little interaction with other electron transport chains, i.e., nitrate reduction occurs mainly by the sequential operation of formate dehydrogenase, cytochrome b_{555}, and nitrate reductase. Severla investigators have demonstrated that NADH may serve as an electron donor for nitrate reduction in cell-free extracts of *E. coli* (2, 5, 6, 13). However, NR$^-$ mutants which lack formate dehydrogenase but possess nitrate reductase neither form nitrite nor remove nitrate from the medium during growth (*unpublished data*). When such mutants reach the stationary phase, however, nitrite begins to accumulate, suggesting that NADH may serve as an electron donor for nitrate reduction only in stationary-phase cells. In contrast, the wild-type parent accumulates large amounts of nitrite during growth under the same conditions, and this accumulation continues during the stationary phase. In none of these cases is nitrite further reduced, presumably because we used 1% nitrate, a condition which suppresses the formation of the nitrite reductase system in *E. coli* (2). The specific role of the NADH-linked nitrate reductase activity and its relationship to nitrate reductase activity associated with formate dehydrogenase is, at present, unknown.

The formate dehydrogenase involved in nitrate reduction appears to be distinct from the formate dehydrogenase involved in the formate hydrogenlyase system. This distinction is based partly on the electron acceptor specificity of the formate dehydrogenase associated with these two systems (5), but it is most clearly demonstrated by the existence of a functional formate hydrogenlyase system and a benzyl viologen-specific formate dehydrogenase in NR$^-$ mutants which lack the phenazine methosulfate-specific formate dehydrogenase associated with nitrate reduction. The fact that some NR$^-$ mutants lack both of these formate dehydrogenases, as

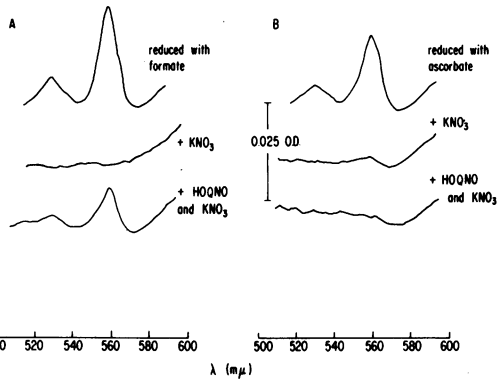

FIG. 5. *Differential spectra of cytochrome b_1 reduced by formate and ascorbate and oxidized by nitrate in the presence of HOQNO. Cells of HfrH were grown anaerobically in complete medium supplemented with 1% potassium nitrate and used as a frozen-thawed suspension. In this case, 10 μg of deoxyribonuclease was added per ml of suspension to reduce the viscosity. Differential spectra were obtained with the split-beam spectrophotometer at room temperature. A sample of cell suspension (3.0 ml containing 2.0 mg of protein per ml) was added to each cuvette. To the reference cuvette, 0.05 ml of 1 M potassium nitrate was added to oxidize the cytochrome b_1. In the reduced cuvettes, 0.05 ml of 0.06 M formate or solid ascorbic acid was added.*

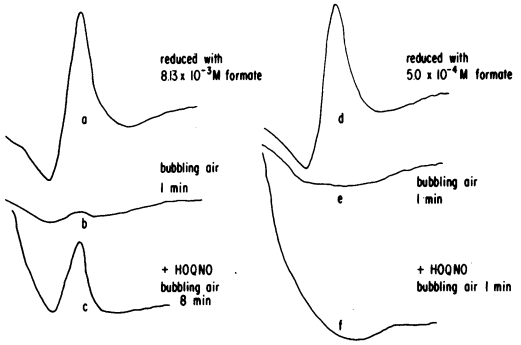

FIG. 6. *Auto-oxidation of cytochrome b_1 and effect of HOQNO. Cells were prepared as in Fig. 4 and used at a protein concentration of 2.3 mg/ml. Difference spectra were determined at room temperature with the split-beam spectrophotometer. The reference cell suspension was oxidized by bubbling with air through a pasteur pipette. (a) Cell suspension plus 8.3 μmoles of sodium formate per ml; (b) suspension (a) bubbled with air for 1 min; (c) suspension (a) plus 8.3×10^{-5} M HOQNO, bubbled with air for 8 min; (d) cell suspension plus 0.5 μmoles of sodium formate per ml; (e) suspension (d) bubbled with air for 1 min; (f) suspension (d) plus 8.3×10^{-3} M HOQNO, bubbled with air for 1 min.*

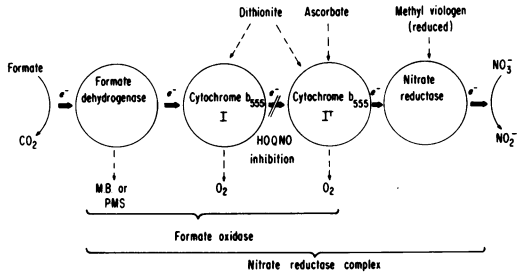

FIG. 7. *Suggested scheme for the nitrate reductase complex of Escherichia coli.*

shown here and by others (14, 22), suggests that both activities depend on a common genetic element, perhaps the structural gene for formate dehydrogenase. If both are specified by the same gene, the genetic and physiological variations observed for the two formate dehydrogenases must result from an interaction and association of the gene product with distinct electron transport components. An alternative interpretation is that both activities are lost as a result of some pleiotropic alteration affecting the membrane components of the cell (1). In any case, a clear understanding of the genetic variations observed for the formate dehydrogenases will require a detailed genetic and biochemical analysis of this enzyme and its participation in distinct pathways.

The scheme in Fig. 7 is proposed to explain the interaction of the nitrate-specific cytochrome b_1 with the different electron donors, its oxidation by nitrate, and the effects of the inhibitor HOQNO on these processes. Several of the facts presented here suggest that two distinct cytochrome b_{555} components are oxidized by nitrate. (i) Nitrate causes a biphasic oxidation of formate-reduced cytochrome b_{555}. (ii) Ascorbate, an electron donor of moderately high redox potential, reduced only a part (about 50%) of the nitrate-specific cytochrome b_{555} which dithionite or formate reduce. (iii) HOQNO inhibits the oxidation of only part (about 50%) of the formate-reduced cytochrome b_{555} by nitrate but does not inhibit the oxidation of ascorbate-reduced cytochrome b_{555} by nitrate. These observations, along with the fact that HOQNO completely inhibits the reduction of nitrate by formate, are most easily explained by the proposal in Fig. 7 that two cytochrome b_{555} components with different oxidation-reduction potentials function sequentially in the transfer of electrons from formate to nitrate. Whereas dithionite and formate (via formate dehydrogenase) would reduce both components, ascorbate would reduce only the second cytochrome component. The selective effects of HOQNO would result from its inhibition of the transfer of electron between the two cytochrome components. The biphasic kinetics of oxidation by nitrate would be due to the ability of formate, as long as it is present, to keep cytochrome I reduced even in the presence of oxidized cytochrome II.

Such a model might explain why many NR^- mutants which lack either nitrate reductase or formate dehydrogenase also exhibit decreased but intermediate levels of the cytochrome b_{555} (17); i. e., one or the other of the two cytochrome components might not be formed. Furthermore, the reports that cytochrome b_1 is associated both with formate dehydrogenase (7, 11) and with nitrate reductase (9) after these enzymes had been resolved from one another can be explained by assuming that one cytochrome b_1 component remains associated with formate dehydrogenase and the other with nitrate reductase during the purification procedures.

It is clear that the nitrate-specific cytochrome b_{555} components are distinct from the cytochrome b_{555} component formed under aerobic conditions, since NR^- mutants which lack the nitrate-induced cytochrome b_{555} still form normal cytochrome b_1 components under aerobic conditions. These observations do not establish whether that is the same cytochrome b_1 associated with different components or that *E. coli* specifies a series of distinct cytochrome b_1 components for various electron transport systems. In any case, the formation of cytochrome components must be specifically regulated by the other catalytic components with which they are normally associated.

That nitrate reduction is catalyzed by a physically associated complex of enzymes can be established only by the isolation of the catalytic unit. However, the membrane association of the components, the existence of pleiotropic mutations affecting the activities, and the apparent lack of interaction of this system with other electron transport chains argue for the existence of such a complex.

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