The Journal of Biochemistry, Vol. 53, No. 5, 1963

Reactions of Cytochrome b_1 and Nitrate Reductase in a Preparation Solubilized from *Escherichia coli**

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(Received for publication, December 25, 1962)

In certain becteria, nitrate can replace molecular oxygen as an oxidant in energy-yielding metabolism. This anaerobic process, called nitrate respiration, differs in its enzymatic mechanisms from nitrate reduction for assimilatory purposes and is rather analogous to aerobic respiration (*1—5*).

The enzyme system involved in nitrate respiration in *Escherichia coli* has been extensively studied (*1, 2, 5—10*), and the following pathway has been proposed for the electron transport from formate, the most effective donor for respiratory nitrate reduction in this organism, to nitrate (*11*):

Formate→formate dehydrogenase**→lipid factor→cytochrome b_1→nitrate reductase→nitrate.

This electron-transport system, like that of aerobic respiration, is tightly bound to the particulate fraction of the cell (*1, 8, 9*), and it has been difficult to obtain its components in soluble states. Taniguchi and Itagaki (*10*) has recently solublized and highly purified nitrate reductase [EC 1. 9. 6. 1] and reported that this enzyme is a metalloprotein containing both molybdenum and non-heme iron. More recently, cytochrome b_1 has also been obtained in a partially purified state and its properties have been reported (*12*). However, the interaction between cytochrome b_1 and nitrate reductase has so far been studied only with particulate preparations. It seems, therefore, desirable to investigate this interaction in a solubilized system in order to confirm further the above-mentioned electron-transport scheme.

In a previous paper (*11*) we reported the solubilization with the aid of snake venom and deoxycholate and partial purification of an enzyme perparation from the particulate fraction of *E. coli*. This soluble preparation contained formate dehydrogenase, cytochrome b_1 and nitrate reductase, but had lost a lipid factor required for the electron transfer from formate dehydrogenase to cytochrome b_1. Although the nature of the lipid factor has not yet been elucidated, vitamin K_3 has been shown to be capable of replacing the natural factor in this system (*11, 13*). The present paper describes some observations on the reactions of cytochrome b_1 and nitrate reductase in this solubilized system. This work has been preliminarily reported elsewhere (*14*).

MATERIALS AND METHODS

Enzyme Preparations—The particulate fraction of *E. coli*, Yamagutchi strain, grown under the conditions of nitrate respiration was prepared as described by Taniguchi and Itagaki (*10*). The enzyme preparation was solubilized therefrom by snake venom and deoxycholate and partially purified as reported by Itagaki *et al.* (*11*). The soluble preparation thus obtained contained formate dehydrogenase (900—1,200 μmoles formate oxidized per hour per mg. of protein; assayed manometrically with methylene blue as acceptor (*15*)), cytochrome b_1 (1—3 mμmoles protoheme per mg. of protein), and nitrate reductase (350 μmoles nitrate reduced per hour per mg. of protein; assayed with reduced methyl viologen as donor (*10*)). These components were present in a form not sedimentable by centrifugation at $105{,}000 \times g$ for 60 minutes.

Chemicals—Vitamin K_3 (3.4 mg.) was dissolved in 4 ml. of acetone and diluted with water to 20 ml. (1 m*M* solution). HOQNO was dissolved in 0.01 *N* NaOH and its concentration was determined from

* 2-Heptyl-4-hydroxyquinoline-N-oxide is abbreviated as HOQNO.

** The identity of the enzyme with formate dehydrogenase [EC 1. 2. 2. 1] is not yet known.

the absorbancy of the solution at 253 mμ according to Jackson and Lightbown (*16*). FAD, FMN and riboflavin were purchased from the Wako Chemical Company, and their concentrations were determined spectrophotometrically using the reported molar extinction coefficients.

Spectrophotometric Experiments—Absorption spectra were measured in a Cary model 14 recording spectrophotometer using cuvettes of 1 cm. light path. The reduction and reoxidation of cytochrome b_1 were followed at 427 mμ, the Soret peak of the reduced cytochrome, also in the Cary spectrophotometer at room temperature (about 20°C). Thunberg-type cuvettes of 1 cm. light path were used for this purpose; they were well evacuated and filled with pure nitrogen. The compositions of reaction mixtures (final volume, 2.0 ml.) are specified in the legends of figures.

Formate-Nitrate Reductase Activity—The reduction of nitrate by formate was measured anaerobically in Thunberg tubes. The reaction mixture contained, in a final volume of 2.0 ml., 10 μmoles each of sodium formate and KNO_3 (in the side arm), and 100 μmoles of Tris-HCl buffer, pH 7.1, the enzyme preparation, and other additions such as vitamin K_3 and HOQNO as indicated in individual experiments (in the main compartment). The reaction was carried out at 37°C for 10 minutes and the nitrite formed was determined as described previously (*10*).

Oxygen Uptake—The oxygen uptake by the particulate fraction was measured in a recording oxygen-electrode apparatus described by Hagihara (*17*). The detailed conditions are given in the legend of Fig. 6.

Protein Determination—Protein was determined by the method of Lowry *et al.* (*18*) using bovine serum albumin as the standard.

RESULTS

Aerobic Oxidation of Reduced Cytochrome b_1—The soluble preparation employed in this study contained sufficient quantities of formate dehydrogenase, cytochrome b_1 and nitrate reductase, but was deficient in the lipid factor required for the reduction of cytochrome b_1 by formate and formate dehydrogenase. As reported previously (*11*, *13*), however, the cytochrome could be fully reduced when a catalytic amount of vitamin K_3 and a substrate amount of formate were added under anaerobic conditions. It was found that the cytochrome reduced anaerobically by the addition of 0.5 m*M* of sodium formate and 50 μ*M* of vitamin K_3 could be rapidly reoxidized when air was bubbled into the reaction mixture. This reoxidation was not inhibited by 20 m*M* of cyanide or azide, indicating that no terminal oxidases are required in this reaction. Spectrophotometrically, it was in fact found that the preparation was completely devoid of cytochromes a_1 and a_2 which are believed to act as the terminal oxidases in this organism (*19*). The autoxidizability of cytochrome b_1 has also been observed with a considerably purified preparation (*12*).

Oxidation of Reduced Cytochrome b_1 by Nitrate—Fig. 1 shows that the cytochrome b_1 in the preparation reduced anaerobically with formate and vitamin K_3 could be reoxidized by the addition of nitrate (final concentration, 5 m*M*) at an initial rate of 4 mμmoles cytochrome oxidized per minute per mg. of pro-

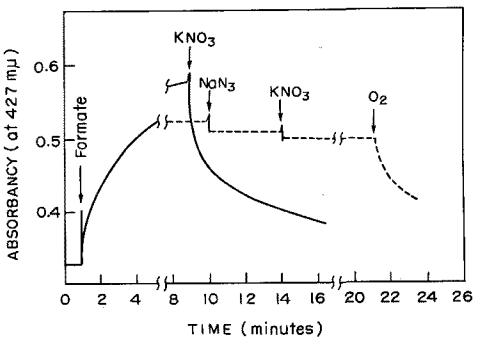

FIG. 1. Reoxidation of reduced cytochrome b_1 by nitrate and by oxygen and effect of azide. The reaction was run anaerobically in Thunberg-type cuvettes. The main compartment contained 1.89 ml. of soluble enzyme preparation (4.8 mg. protein) in phosphate buffer (pH 7.1) containing 75 mμmoles vitamin K_3, and the side arm received 0.01 ml. of sodium formate (1 μmole). After evacuation, the tube was filled with pure nitrogen, and the reduction of cytochrome b_1 was started by tipping formate into the main compartment. At indicated time, the tube was quickly opened and 0.1 ml. of KNO_3 (10 μmoles) or NaN_3 (20 μmoles) was added followed by rapid evacuation and refilling of nitrogen gas. Pure oxygen was bubbled through a capillary for 20 seconds as indicated.

tein. This reoxidation was accompanied by the accumulation of nitrite in the reaction medium. The absorption spectrum of the preparation had returned to that of the aerobically oxidized preparation after the completion of the reaction (Fig. 2). As shown in

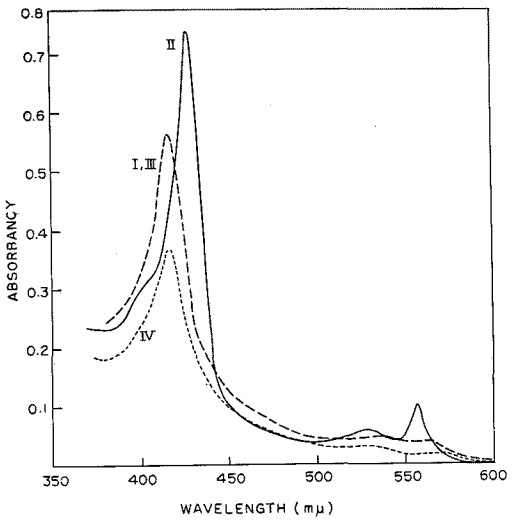

FIG. 2. Absorption spectra of cytochrome b_1 reoxidized by nitrate and by chlorate. The soluble preparation (2.5 mg. protein) in a final volume of 2.0 ml. of 25 m*M* Tris-HCl buffer (pH 7.1) was placed in a Thunberg-type cuvette and anaerobically treated with 1 μmole of sodium formate and 50 mμmoles of vitamin K_3. The reduced cytochrome b_1 thus produced was then reoxidized by anaerobic addition of 10 μmoles of KNO_3 or $KClO_3$. The spectra of reoxidized cytochrome were measured 25 minutes after the addition of the oxidizing reagents. (I) Oxidized form; (II) reduced form; (III) preparation reoxidized by nitrate; (IV) preparation reoxidized by chlorate.

Fig. 1, this reoxidation by nitrate was completely inhibited by azide (10 m*M*). Cyanide was also an effective inhibitor. In view of the unreactivity of cytochrome b_1 with azide and cyanide (*11*, *12*), these findings seems to indicate the involvement of a factor sensitive to these reagents in the reaction between reduced cytochrome b_1 and nitrate. Since the activity of highly purified nitrate reductase, as measured with reduced methyl viologen as a donor, is strongly inhibited by both cyanide and azide (*10*) it may be concluded that this enzyme is involved in the observed oxidation of cytochrome b_1. Owing to its autoxidizability, the reduced cytochrome could be rapidly reoxidized by oxygen even in the azide-inhibited system (Fig. 1).

Oxidation of Reduced Cytochrome b_1 by Chlorate—Chlorate has been shown to inhibit competitively the reduction of nitrate by an extract of *E. coli* (*20*, *21*) and by purified nitrate reductase (*5*). It has further been revealed that chlorate itself can be reduced by nitrate reductase*. The effect of chlorate on reduced cytochrome b_1 was therefore examined in the soluble preparation. It was thus found, as is shown in Fig. 3, that chlorate, like ni-

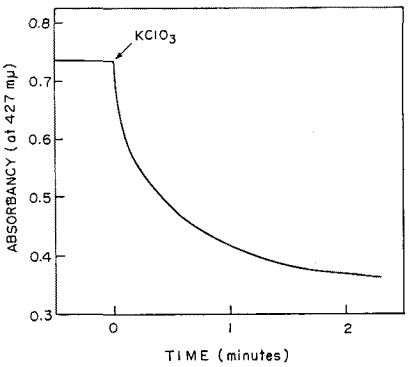

FIG. 3. Reoxidation of cytochrome b_1 by chlorate. In the main compartment of a Thunberg-type cuvette was placed 1.90 ml. of the soluble preparation (2.5 mg. protein) in 25 m*M* Tris-HCl buffer (pH 7.1) containing 10 μmoles of sodium formate and 50 mμmoles of vitamin K_3. The side-arm received 0.1 ml. of $KClO_3$ (10 μmoles). The system was anaerobically incubated at room temperature until the cytochrome became fully reduced, and then the chlorate was added to the main compartment.

trate, could rapidly reoxidize the reduced cytochrome. The initial rate of this reoxidation was 11 mμmoles cytochrome oxidized per minute per mg. of protein when the final concentration of added chlorate was 5 m*M*.

* E. Itagaki and S. Taniguchi, unpublished observation.

Both cyanide and azide were again strongly inhibitory toward this oxidation, and the reduced form of a purified cytochrome b_1, free from nitrate reductase (*12*), was found not to be reoxidizable by chlorate*. It is, therefore, clear that nitrate reductase is also responsible for this oxidation.

It was, however, found, that the absorption spectrum of the preparation after oxidation by chlorate was different from that of normal oxidized cytochrome. As shown in Fig. 2, the Soret peak of the spectrum thus obtained was lower by more than 30 per cent than that of the oxidized spectrum. It is likely that this had been caused by the oxidative deformation of the heme by chlorite, the product of chlorate reduction.

Vitamin K_3 Requirement for Formate-Nirate Reductase Activity—Table I shows that the formate-nitrate reductase activity of the soluble preparation was negligibly low in spite of the presence of formate dehydrogenase and nitrate reductase in sufficient quantities. An active reduction of nitrate by formase was, however, observed when 100 μM of vitamin K_3 was added. The concentration of vitamin K_3 giving half maximal activity was 50 μM (see Fig. 4). These findings provide another support for the participation of cytochrome b_1 in the nitrate-reducing system, since, as mentioned above, the cytochrome in this preparation could be actively reduced only when supplemented with vitamin K_3. Table I further shows that FAD, FMN and riboflavin could not stimulate the formate-nitrate reductase activity even in the presence of vitamin K_3. This may be taken to suggest that the flavin prosthetic group of formate dehydrogenase (*11*) has not been lost during solubilization and purification of the preparation.

HOQNO Inhibition in Soluble System—In agreement with the results obtained with particulate preparations (*1*, *9*), the formate-nitrate reductase activity of the vitamin K_3-supplemented soluble preparation was found to be strongly inhibited by 22 μM of HOQNO as shown in Table II. The very low activity in the absence of vitamin K_3 was also depressed. Although de Heredia and Medina (*22*) have reported a competitive response of vitamin K_3 toward the HOQNO inhibition of the $NADH_2$-nitrate reductase activity of a crude extract of *E. coli*, such competition could not be confirmed in the present system. The inhibition in the present system was actually non-competitive with respect to the vitamin K_3 concentration as will be seen from Fig. 4.

TABLE I

Effects of Vitamin K_3 and Flavins on the Formate-Nitrate Reductase Activity of the Soluble Preparation

The reaction was carried out anaerobically at 37°C. The reaction mixture contained: (in side arm) 0.1 M sodium formate, 0.1 ml.; 0.1 M KNO_3, 0.1 ml.; (in main compartment) soluble enzyme preparation, 0.26 mg. of protein; 0.5 M Tris-HCl buffer (pH 7.1), 0.2 ml. and 1 M flavins, 0.1 ml. or 1 mM vitamin K_3, 0.2 ml. as indicated. The final volume was 2.0 ml.

Additions	Nitrite formed (μmole/hour)
None	0.04
Vitamin K_3	2.52
Riboflavin	0.09
FMN	0.09
FAD	0.06
Vitamin K_3+FMN	2.37
Vitamin K_3+FAD	2.25

* T. Fujita, unpublished observation.

TABLE II

Inhibition by HOQNO of the Formate-Nitrate Reductase Activity of the Soluble Preparation

The reaction conditions were the same as specified in Table I, except that 0.1 mg. protein of the enzyme preparation was used. The final concentration of vitamin K_3 and HOQNO were 25 μM and 22 μM, respectively.

Additions	Nitrite formed (μmole/hour)
None	0.04
HOQNO	0.03
Vitamin K_3	0.68
Vitamin K_3+HOQNO	0.05

It appears, therefore, that HOQNO reacts with the electron-transport system at a site other than that connected with vitamin K_3.

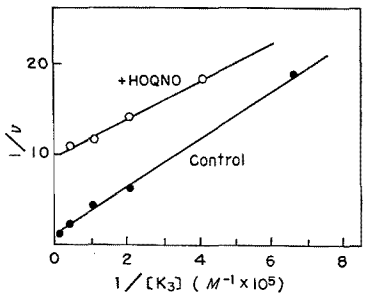

FIG. 4. Effect of vitamin K_3 concentration on HOQNO inhibition of the formate-nitrate reductase activity of the soluble preparation. The reaction mixture contained, in a final volume of 2.0 ml., enzyme preparation (0.25 mg. protein), 10 μmoles each of sodium formate and KNO_3, 50 μmoles of Tris-HCl buffer (pH 7.1), indicated amounts of vitamin K_3, and 0.2 ml. of HOQNO (8.8 mμmoles) in 0.01 *N* NaOH or 0.01 *N* NaOH. The reaction was carried out anaerobically at 37°C for 10 minutes. The reaction velocity, *v*, was expressed in terms of μmoles of nitrite formed per hour. The data are plotted according to Lineweaver and Burk. —●—, Without HOQNO; —○—, HOQNO added.

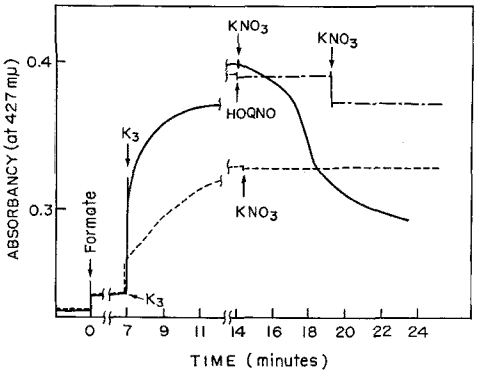

FIG. 5. Effects of HOQNO on reduction by formate plus vitamin K_3 and reoxidation by nitrate of cytochrome b_1. The reaction was conducted anaerobically in Thunberg-type cuvettes and the anaerobic additions were done as described in the legend of Fig. 1. The final reaction mixture contained,in 2.0 ml. of 25 m*M* Tris-HCl buffer (pH 7.1), the soluble preparation (2.44 mg. protein), 1 μmole of sodium formate, 50 mμmoles of vitamin K_3, 5 μmoles of KNO_3, and, when indicated, 88 mμmoles of HOQNO. ——, Control (without HOQNO); ----, HOQNO was present in the reaction mixture throughout the experiment; ·····; HOQNO was added after complete reduction of cytochrome b_1.

Spectrophotometric expriments, illustrated in Fig. 5, showed that the vitamin K_3-mediated reduction of cytochrome b_1 by formate in the solubilized preparation as well as the slow reduction in the absence of vitamin K_3 were insensitive to 44 μM of HOQNO, a concentration capable of inhibiting almost completely the formate-nitrate reaction. The reoxidation of reduced cytochrome b_1 by nitrate, on the other hand, was strongly inhibited by the inhibitor. These findings are in contrast to those of Jackson and Lightbown (*16*) who have reported that this inhibitor inhibits the electron-transport chain of *E. coli* by blocking the reduction of cytochrome b_1. It was futher found that the aerobic oxidation of reduced cytochrome b_1 is not affected by HOQNO in the soluble preparation.

HOQNO Inhibition in Particulate System—In order to further elucidate the mechanism of HOQNO inhibition of the electron-transport reactions in *E. coli*, the effects of this inhibitor on the formate oxidase and formate-nitrate reductase activities of the particulate fraction were examined. As shown in Fig. 6, it was found that the reduction of nitrate by formate is much more sensitive to HOQNO than the aerobic oxidation of formate. While 8 to 9 μM of HOQNO was sufficient to cause a 50 % inhibition of the formate-nitrate reaction, about 120 μM of the inhibitor was required to inhibit the formate-oxygen reaction to the same extent. It is interesting to note that the formate oxidase activity of the particulate fraction was about 25% inhibited by 38 μM of HOQNO, whereas, as mentioned above, the aerobic oxidation of reduced cytochrome b_1 in the solubilized system was not inhibited at all by 44 μM of the inhibitor.

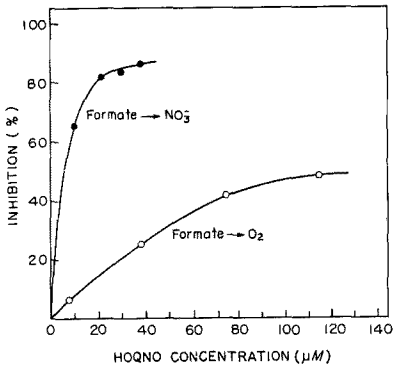

FIG. 6. Effects of HOQNO on the formate oxidase and formate-nitrate reductase activities of the particulate preparation. The formate oxidase activity was measured by following oxygen uptake in a recording oxygen-electrode apparatus at 22°C. The reaction mixture contained, in a final volume of 2.0 ml., the particulate preparation (5.4 mg. protein), 100 μmoles of Tris-HCl buffer (pH 7.1), 10 μmoles of sodium formate, and indicated amounts of HOQNO (in 0.02 *N* NaOH). The reaction was started by adding formate. The formate-nitrate reductase activity was measured as described in the "Materials and Methods" section. —○—, Formate oxidase; —●—, formate-nitrate reductase.

DISCUSSION

The participation of cytochromes of the *b* or *c* type in the respiratory reduction of nitrate, nitrite and hydroxylamine has been reported in certain microorganisms (*5*, *23*, *24*). Furthermore, interactions in solubilized systems between cytochromes of the *c* type and nitrite or hydroxylamine reductase have been studied with preparations from a halotolerant micrococcus (*23*), *Micrococcus denitrificans* (*25*) and *Pseudomonas aeruginosa* (*26*). However, the reaction of cytochrome b_1 with nitrate reductase in *E. coli*, though supported by ample evidence, has not yet been confirmed in a solubilized system. In the present work, in which a preparation solubilized and partially purified from the particulate fraction of *E. coli* cells was employed, it was shown that reduced cytochrome b_1 can be anaerobically reoxidized by nitrate in a cyanide- and azide-sensitive reaction. The reduced cytochrome in this system could also be reoxidized by chlorate, which is a competitive inhibitor of nitrate reduction (*20*, *21*) and is itself reducible by purified nitrate reductase (*5*). These findings clearly indicate that there is in fact an interaction between cytochrome b_1 and nitrate reductase.

The requiremet of vitamin K_3 for the reduction of nitrate by formate in the solubilized system seems to further support the participation of cytochrome b_1 in the nitrate-reducing mechanism. As already discussed, this requirement can be explained by the absence from the preparation of a lipid factor functionally connecting formate dehydrogenase (and possibly also the other particulate-bound flavoprotein enzymes) to cytochrome b_1 and by the ability of vitamin K_3 to replace the natural factor. The reported occurrence in *E. coli* of a $NADH_2$-vitamin K_3 reductase activity (*27*) may be related to this lipid factor. It is of interest in this connection that both the formate-nitrate and $NADH_2$-nitrate reductase activities of the particulate fraction of *E. coli* have been reported to be stimulated more than two-fold by the addition of vitamin K_3 (*9*, *22*, *28*). This fact might be regarded to indicate that some of the lipid factor has been lost from the particulate fraction during the disruption of cells and centrifugal fractionation.

HOQNO has been shown to inhibit strongly the respiratory electron-transport chains in heart-muscle preparation (*16* *29*) and several microbial cells (*16*). The photophosphorylation reactions in both bacterial chromatophores (*30*) and green-plant chloroplasts (*31*, *32*) have also been shown to be highly sensitive to this inhibitor. However, the precise mechanisms of these inhibitions have not yet been well defined (see ref. *33*). Previous studies have shown that the formate-nitrate reductase system of the particulate fraction of *E. coli* (*1*, *9*) as well as the $NADH_2$-nitrate reductase activity in crude extracts of the same organism (*22*) are also highly HOQNO-sensitive. In the present study, the strong inhibition by HOQNO of the formate-nitrate reaction was confirmed in a solubili-

zed system. In contrast to the observations of de Heredia and Medina (*22*) on the $NADH_2$-nitrate reaction in *E. coli* extracts, however, it was not possible to show competition between HOQNO and vitamin K_3. The site of HOQNO inhibition, therefore, must be somewhere other than the vitamin K_3-site.

Jackson and Lightbown (*16*) have reported that in *E. coli* HOQNO prevents the respiration by inhibiting the reduction of cytochrome b_1. In heart-muscle preparations and in certain bacteria such as *Bacillus subtilis* and *Staphylococccus aureus*, on the other hand, the oxidation (but not the reduction) of *b*-type cytochromes is the reaction inhibited by HOQNO (*16*). It is, however, revealed in the present study that in the solubilized system obtained from *E. coli* HOQNO prevents the reoxidation of reduced cytochrome b_1 by nitrate rather than the reduction of the pigment by formate. Since the activity of nitrate reductase as measured with reduced methyl viologen as donor is insensitive to this inhibitor (*10*), this inhibition can not be accounted for by the sensitivity of this enzyme to HOQNO. It was further shown that the autoxidation of reduced cytochrome b_1 in the solubilized preparation is insensitive to the inhibitor.

These experimental findings obtained with the solubilized preparation seems to be explicable by assuming the intervention of a HOQNO-sensitive factor between cytochrome b_1 and nitrate reductase. Such hypothetical factor, if present at all, might be of lipophilic nature or, according to Tappel's suggestion (*34*), might represent the lipid molecular environments around cytochrome b_1 or nitrate reductase. Another possible mechanism of HOQNO inhibition is that the inhibitor does combine with reduced cytochrome b_1 and thus prevents its interaction with nitrate reductase. If this be the case, however, it must be assumed that the HOQNO-cytochrome b_1 complex is as autoxidizable as the uncomplexed cytochrome, since the autoxidation of the reduced cytochrome is insensitive to the inhibitor. It should also be noted in this connection that no spectral evidence is available for the formation of such a complex.

Experiments with the particulate preparation further indicated that the formate-nitrate reductase activity is much more sensitive to HOQNO than the formate oxidase activity. Since the electron-transport pathway from formate to cytochrome b_1 is most probably common for both nitrate reduction and oxygen uptake, this selective inhibiction by HOQNO again suggests that the inhibition site is not situated in the range from formate to cytochrome b_1. The fact that the formate oxidase activity of the particulate fraction is inhibited by HOQNO, though to much less extents than the nitrate reduction process, is of interest in view of the insensitivity of the autoxidation of reduced cytochrome b_1 to the inhibitor. Apparently, this suggests that in the particulate system cytochrome b_1 is not appreciably autoxidizable and its reoxidation by oxygen requires the intervention of a HOQNO-sentitive component. However, it is not yet clear if this component is identical with cytochrome a_1 or a_2, the components of terminal oxidase system of *E. coli*. At any rate, the site lying between cytochrome b_1 and oxygen is much less sensitive to HOQNO than that between cytochrome b_1 and nitrate reductase. This low sensitivity of the oxidase site is in accord with the finding that the respiration of *E. coli* and *Proteus vulgaris* is much more resistant to HOQNO as compared with that of heart-muscle preparations and such bacteria as *B. subtilis* and *S. aureus* (*16*, *33*).

In conclusion, it is still premature to draw any decisive conclusions concerning the mechanesm of HOQNO inhibition of the electron-transport reactions in *E. coli*. This seems especially so in view of the complicated behaviors of this inhibitor toward certain oxidation-reduction reactions as reported by Kogut and Lightbown (*33*).

The earlier observations that reduced cytochrome b_1 in the solubilized state is fairly strongly autoxidizable (*11—13*) were confirmed also in the present study. Since the cytochrome in the solubilized system is unable to bind carbon monoxide (*11*, *12*), this autoxidizability does not seem to indicate the dena-

turation of the hemoprotein during solubilization. The reduced cytochrome in the particulate fraction of *E. coli*, on the other hand, does not appear to be appreciably autoxidizable, since its reoxidation by molecular oxygen requires the presence of terminal oxidases (probably cytochromes a_1 and a_2) and is inhibited by cyanide, azide and HOQNO. In the intact particulate structure, therefore, cytochrome b_1 seems to be protected in some ways from the direct attack by molecular oxygen.

The nitrate reductase-catalyzed reoxidation of reduced cytochrome b_1 by chlorate can be explained by the structural similarity between nitrate and chlorate as suggested previously (*21*). The results reported in this paper, however, show that the heme moiety of cytochrome b_1 thereby undergoes certain degradation probably due to the powerful oxidative activity of chlorite, the product of chlorate reduction. The toxic effect of chlorate on the growth of microorganisms and higher plants (*35—37*) may, therefore, be caused not only by the competitive inhibition of nitrate reduction but also by oxidative destruction of essential cellular components by chlorite and possibly also hypochlorite produced in chlorate reduction.

SUMMARY

The reactions of cytochrome b_1 and nitrate reductase in an enzyme preparation solubilized and partially purified from the particulate fraction of *Escherichia coli* were investigated. The cytochrome b_1 reduced by the addition of formate and vitamin K_3 was found to be reoxidizable by oxygen, nitrate and chlorate. While the oxidation by oxygen was insensitive to cyanide and azide (the autoxidizability of the cytochrome), these reagents strongly inhibited the reaction by nitrate and chlorate suggesting the intervention of nitrate reductase. With chlorate as the oxidant, the deformation of the absorption spectrum of the cytochrome was observed. The nitrate reduction by formate in the solubilized system was found to require the addition of vitamin K_3, but flavins had no effects on this reaction. Since the vitamin is required also for the reduction of cytochrome b_1 in this preparation, the involvement of the cytochrome in the nitrate-reducing mechanism was further substantiated. The formate-nitrate reaction was strongly inhibited by 2-heptyl-4-hydroxyquinoline-N-oxide (HOQNO), but this inhibition was not competitive with respect to vitamin K_3. It was spectrophotometrically revealed that HOQNO inhibits the reoxidation of cytochrome b_1 by nitrate, but not the reduction by formate plus vitamin K_3. In the particulate fraction, the formate-nitrate reductase activity was inhibited much more remarkably than the formate oxidase activity. Some of the implications of these results are discussed.

We wish to thank Dr. S. Taniguchi for a generous gift of 2-heptyl-4-hydoxyquinoline-N-oxide.

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