Pyruvate:flavodoxin oxidoreductase, the nifJ gene product of Klebsiella pneumoniae, was purified to homogeneity. Pyruvate:flavodoxin oxidoreductase, flavodoxin, and nitrogenase components I and II are the only proteins required for pyruvate-coupled nitrogenase activity. The physiological source of electrons to nitrogenase in K. pneumoniae is pyruvate. Flavodoxin from Azotobacter vinelandii was only one-third as effective as K. pneumoniae flavodoxin in transferring electrons from pyruvate:flavodoxin oxidoreductase to Azotobacter and Klebsiella nitrogenases. Ferrodoxins from aerobic, anaerobic and photosynthetic nitrogen-fixing organisms, as well as benzyl viologen and methyl viologen, were ineffective in coupling pyruvate oxidation to nitrogenase activity. One mol each of acetyl-CoA, CO₂, and ethylene are formed by pyruvate-supported acetate reduction. The enzyme contains 8.0 ± 0.6 mol of iron and 6.6 ± 0.2 mol of acid-labile sulfide per mol of protein (M₀ = 240,000). Pyruvate:flavodoxin oxidoreductase is irreversibly inactivated by air.

The protein products of two genes, nifF and nifJ, have been implicated in electron transport to nitrogenase in Klebsiella pneumoniae (1, 2). NifF⁻ and NifJ⁻ mutant strains have no nitrogenase activity in vivo, but have activity in vitro with sodium dithionite as the electron donor (1, 2). Dithionite reduces nitrogenase directly (3), thus bypassing the low potential electron donor required for nitrogen fixation in vivo. Crude extracts from wild type K. pneumoniae can couple the oxidation of malate, pyruvate, or formate to nitrogenase activity (4), whereas extracts from NifF⁻ and NifJ⁻ mutant strains fail to establish this coupling (5, 6). Electron flow is established when a mixture of NifF⁺ and NifJ⁺ extracts were used (5, 6). The nifF gene product is a flavoprotein (5), called flavodoxin. The nifJ gene product consists of two identical subunits (7, 8), each having a molecular weight of 120,000 (2, 9, 10). Bogusz et al. (7) claimed to have purified the nifJ gene product, which contained 30 mol of iron and 24 mol of acid-labile sulfur per mol of protein.

We report the purification of the nifJ gene product, a pyruvate:flavodoxin oxidoreductase, having properties quite different from that reported by Bogusz et al. (7). Electron transport to nitrogenase was established with purified protein components.

**Experimental Procedures**

**Materials**

ATP, creatine phosphate, dithiothreitol, glutathione, thiamin pyrophosphate, coenzyme A, sodium pyruvate, S-acetyl coenzyme A, NAD, L-malate, glycerol, α-ketoglutaric acid, malic dehydrogenase, citrate synthase, Hyamine hydroxide, Tris base, and deoxyribonuclease I were obtained from Sigma. Creatine phosphokinase was obtained from Miles Laboratories, Elkhart, IN. DEAE-cellulose was a Whatman DE52 (microgranular) product. Sephadex S-200 and Sephadex G-50 were obtained from Pharmacia Fine Chemicals, Piscataway, NJ. Hydroxylapatite used was Bio-Gel HTP from Bio-Rad Laboratories. Sodium [1-¹⁴C]pyruvate and Bray’s solution were purchased from New England Nuclear. The solution of radioactive pyruvate was prepared in 0.01 N HCl. All other chemicals and gases were of analytical grade available commercially.

**Methods**

**Bacterial Strains and Growth Conditions**—K. pneumoniae strain M5al (UN) is the wild type. Mutant strains UN1108 (nifJ4428) and UN3409 (nifJ5520) have been described (11). The basal medium, described previously (5), was used as the minimal medium. Cells were grown anaerobically without shaking for 16-18 h at 30 °C in 250 ml of medium containing 28 mM ammonium acetate. Cells were harvested and resuspended in 500 ml of nitrogen-free medium. After incubation for 1.5 h anaerobically, L-serine was added to a final concentration of 0.4 mM. The cells were incubated for an additional 4.5 h and harvested under an argon atmosphere. The cells were washed by suspending them in N₂-sparged 0.1 M Tris-HCl buffer, pH 7.4, containing 0.86 mM sodium dithionite, followed by centrifugation. The cell paste was stored at −80 °C under an argon atmosphere. For large scale preparations, 18-liter carboys were used and the cells were harvested with a Sharples centrifuge. The cell paste was frozen in liquid nitrogen and stored at −80 °C in a gas-tight container.

**Preparation of Crude Extract**—All buffers used throughout the purification of pyruvate:flavodoxin oxidoreductase were sparged with prepurified nitrogen and contained 20% glycerol unless otherwise specified. These buffers were further deoxygenated by repeated evacuation and flushing with argon purified through a heated copper catalyst. These buffers contained 5 mM dithiothreitol, 1 mM glutathione, 1 mM MgCl₂, 0.05 mM thiamin pyrophosphate, and 0.86 mM sodium dithionite, added just before use.

The cells were suspended in 0.1 M Tris-HCl buffer, pH 7.4 (without glycerol), at a concentration of 1 g of cell paste per 2 ml of buffer containing 5-10 μg of deoxyribonuclease I/ml. The cells were broken anaerobically with a French pressure cell at 16,000 psi and centrifuged at 31,000 × g for 40 min at 0-4 °C under an argon atmosphere. The supernatant solution was transferred to an argon-filled bottle with syringes that had been flushed with argon. The crude extract was stored at −20 °C. The pellets of broken cells were pooled and stored at −20 °C for the isolation of flavodoxin.

**Pyruvate:Flavodoxin Oxidoreductase-coupled Nitrogenase Assay**—The assays were carried out in 9-ml serum vials (1 ml of reaction mixture) containing the following, unless otherwise specified. 25 mM Tris-HCl (pH 7.4), 2.5 mM ATP, 20 mM creatine phosphate, 5 mM...
Pyrophosphate, 2 mM dithiothreitol, 0.4 mM coenzyme A, and 25 µg of nitrogenase component II were added and the vials were incubated for 5 min to use up traces of dithionite present. The vials were brought to atmospheric pressure by piercing the serum stoppers with a hypodermic needle. One to ten µl of pyruvate:flavodoxin oxidoreductase and 30 µl of NaOH were injected and the vials were incubated at 30°C for 15 min in a water bath shaker. The reaction was terminated by injecting 0.1 ml of 4 mM NaOH and the ethylene formed was measured with a Packard gas chromatograph with a Porapak N column. Specific activities are expressed as nanomoles of ethylene formed per min per mg of protein.

One unit of pyruvate:flavodoxin oxidoreductase is defined as the amount required to produce 1 nmol of ethylene per min under the assay conditions. All reagents were stored in sealed containers under an argon atmosphere. All additions to sealed serum vials were made with microliter syringes flushed with prepurified argon. Because dithionite is used to protect oxygen-labile components, control assays without pyruvate, coenzyme A, or flavodoxin were carried out with each set of assays to ensure that dithionite has not contributed to any observed activity.

**Purification of Pyruvate:Flavodoxin Oxidoreductase**—All operations were carried out under anaerobic conditions (94% N2, 6% CO2. 0°C) in a 250-ml cell culture column (2.5 x 32 cm) with 30 ml of 25 mM Tris-HCl (pH 7.4) containing 0.86 mM dithionite was repeated and the last traces of oxygen. 10 mM sodium pyruvate, 0.05 mM thiamin pyrophosphate, 0.25 mM MgCl2, 0.2 mg of creatine phosphokinase, and 12 µl of 25 mM Tris-HCl (pH 7.4) was applied to a DEAE-cellulose column (2.5 x 32 cm) equilibrated with 0.2 M NaCl in 0.025 M Tris-HCl, pH 7.4. The column was washed with one bed volume of 0.2 M NaCl in 0.025 M Tris-HCl before eluting flavodoxin with 0.35 M NaCl in the same buffer. The fractions containing flavodoxin were pooled, concentrated in an ultrafiltration cell with a PM10 membrane, and applied to a Sephadex G-50 column (2.5 x 5 cm) equilibrated with 0.2 M NaCl in 0.025 M Tris-HCl, pH 7.4. The column was eluted at a flow rate of 15 ml/h and 2-ml fractions were collected. The most active fractions were pooled, concentrated, and purified to homogeneity by preparative polyacrylamide gel electrophoresis (5, 12).

**Reduction and Oxidation of Flavodoxin**—Flavodoxin absorption spectra were recorded with a Beckman model 25 spectrophotometer in 1 ml of reaction mixture. The experimental cuvette contained 25 mM Tris-HCl (pH 7.4), 2.5 mM ATP, 30 mM creatine phosphate, 5 mM MgCl2, 0.2 mg of creatine phosphokinase, 2 mM sodium pyruvate, 0.01 mM thiamin pyrophosphate, 80 µM coenzyme A, 0.4 mM dithiothreitol II, and 0.5 mM flavodoxin from K. pneumoniae. The reference cuvette contained all components except flavodoxin. Cuvettes were sealed with rubber stoppers and repeatedly evacuated and filled with prepurified argon. The spectrum of flavodoxin was recorded against the reference. Fifty µg of nitrogenase component I and 5 µg of pyruvate:flavodoxin oxidoreductase were added to both cuvets. The spectrum of flavodoxin was recorded after 10 min. Twenty-five µg of nitrogenase component II was added to both cuvets and the spectrum was recorded after 10 min.

**Determination of CO2—CO2 formed from 1-14Cpyruvate was assayed in a 13.5-ml serum vial with a center well (13). The outer well contained 1 ml of reaction mixture for pyruvate-supported nitrogenase activity (mentioned earlier), except that the pyruvate concentration was decreased to 1.8 mM and 0.2 mM [1-14Cpyruvate was included in the assay. The center well contained 0.2 ml of Hymamine hydroxide solution. Pyruvate-coupled nitrogenase assays were carried out in 0.2 ml of reaction mixture of pyruvate-supported nitrogenase for 15 min at 30°C in a water bath shaker. The control assay contained all components except pyruvate:flavodoxin oxidoreductase. One-tenth ml of 20% perchloric acid was injected to the outer well to stop the reaction and the vials were incubated in the shaker for 20 min. To assure complete removal of 14CO2 formed, 0.2 ml of 1 M NaHCO3 was injected to the outer well and the shaking was continued for an additional 20 min. Following ethylene estimations, the Hymamine hydroxide solution was counted in 5 ml of Bray's solution (14), using a Packard scintillation counter.

**Determination of Acetyl-CoA—Acetyl-CoA was determined with monitoring of Hymamine hydroxide solution (13). Pyruvate-supported nitrogenase assays were carried out as described earlier. One-tenth ml of 20% perchloric acid was injected in the vials to stop the reaction. Following ethylene estimations, the vials were centrifuged at 600 x g for 10 min. The supernatant solution was transferred to a centrifuge tube, neutralized with 2 X KOH, and centrifuged again. Two-tenths ml of the supernatant solution was used for the determination of acetyl-CoA. Under the assay conditions, 10 nmol of acetyl-CoA produced 0.049 absorbance change at a wavelength of 340 nm.

**K. pneumoniae and Azotobacter vinelandii nitrogenase components I and II** were purified as previously reported (15, 12). A. vinelandii flavodoxin, obtained as a side product during component II purification (12), was further purified by the same method used for the purification of K. pneumoniae flavodoxin. Protein concentrations were determined by the method of Lowry et al. (16), using bovine serum albumin as a standard. Iron (17) and acid-labile sulfide (18) contents were determined by published methods. Standard iron solution was obtained from Fisher. Glass-distilled water was used throughout the purification and acid-cleaned glassware were used where necessary. SDS-polyacrylamide gel electrophoresis was performed by slab-gel modification (19) of the Laemmli method (20), using 10% acrylamide.

**RESULTS AND DISCUSSION**

The results of a representative purification of pyruvate:flavodoxin oxidoreductase are shown in Table I. The enzyme was purified about 47-fold with 17% recovery of the enzyme in the extract of a NifF mutant (UN3409) with pyruvate as an electron source under the conditions described earlier. NifF mutant extract was the source of pyruvate:flavodoxin oxidoreductase, component I and component II in these assays. Active fractions were diluted with an equal volume of 0.025 M Tris-HCl (pH 7.4) and applied to a DEAE-cellulose column (2.5 x 30 cm) equilibrated with 0.025 M Tris-HCl, pH 7.4. The column was washed with one bed volume of 0.2 M NaCl in 0.025 M Tris-HCl before eluting flavodoxin with 0.35 M NaCl in the same buffer. The fractions containing flavodoxin were pooled, concentrated, and eluted with prepurified argon. The spectrum of flavodoxin was recorded against the reference. Fifty µg of nitrogenase component I and 5 µg of pyruvate:flavodoxin oxidoreductase were added to both cuvets. The spectrum of flavodoxin was recorded after 10 min. Twenty-five µg of nitrogenase component II was added to both cuvets and the spectrum was recorded after 10 min.

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**RESULTS AND DISCUSSION**

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The abbreviation used is: SDS, sodium dodecyl sulfate.
activity present in crude extract. SDS-polyacrylamide gel electrophoresis of the enzyme showed a single band upon staining with Coomassie blue (Fig. 1). It can be seen from this figure that the 120,000-dalton band is present in the wild type *K. pneumoniae* extract, but is missing from an extract of a NifJ- mutant. This polypeptide was previously demonstrated, with the use of mutants, to be coded by the nifJ gene (2, 9, 10). The identification of purified pyruvate:flavodoxin oxidoreductase as the *nifJ* gene product was further confirmed by reconstituting pyruvate-supported nitrogenase activity in the extract of NifJ- mutant (5, 6). Pyruvate:flavodoxin oxidoreductase is extremely sensitive to oxygen. A 5-min exposure to air inactivated the enzyme and addition of dithionite did not restore the activity. In crude extracts, pyruvate:flavodoxin oxidoreductase is reasonably stable to freezing and thawing, but the purified enzyme is much more susceptible.

The pyruvate:flavodoxin oxidoreductase contains 8.0 ± 0.6 mol of iron and 6.6 ± 0.2 mol of acid-labile sulfide per mol of active enzyme (*M₅ = 240,000*). The only other purified pyruvate:flavodoxin oxidoreductase reported (21) was isolated from *Escherichia coli*. This enzyme was partially purified, but its metal composition was not investigated. Pyruvate:ferredoxin oxidoreductase from *Clostridium acidi-urici* (13) contains 6 mol of iron and 3 mol of acid-labile sulfide and, that from *Halobacterium halobium* (22), 7.6 mol of iron and 5.2 mol of acid-labile sulfide.

Although malate, formate, pyruvate, NADPH-regenerating system, and glucose 6-phosphate have been used as sources of reducing power for *K. pneumoniae* nitrogenase in crude extracts (4–6), the purified enzyme failed to show any activity with substrates other than pyruvate. α-Ketoglutarate was ineffective in these assays (Table I). These data support the idea that pyruvate is the only direct physiological substrate of electrons to nitrogenase in *K. pneumoniae*. E. coli (21) pyruvate:flavodoxin oxidoreductase is ineffective with α-ketoglutarate, whereas *H. halobium* (22) pyruvate:ferredoxin oxidoreductase accepts pyruvate, α-ketoglutarate, and α-ketobutyrate as substrates.

The requirements for pyruvate:flavodoxin oxidoreductase-coupled nitrogenase activity are presented in Table II. Omission of any one of the components of the assay system prevented nitrogenase activity. Pyruvate-coupled nitrogenase activity was stimulated 3- to 4-fold by the addition of thiamin pyrophosphate to crude extracts of *K. pneumoniae*. Thiamin pyrophosphate was added to all the buffers used for purification of pyruvate:flavodoxin oxidoreductase to protect the enzyme (13), hence we could not test its requirement with purified pyruvate:flavodoxin oxidoreductase. It is possible that thiamin pyrophosphate is an enzyme-bound cofactor of pyruvate:flavodoxin oxidoreductase, as reported for pyruvate:ferredoxin oxidoreductases (15, 22).

To test its specificity for electron carrier, the pyruvate:flavodoxin oxidoreductase was assayed with a variety of low potential electron carriers (Table III). The pyruvate:flavodoxin oxidoreductase is specific to Klebsiella flavodoxin. At comparable concentrations, *A. vinelandii* flavodoxin was only one-third as effective as *K. pneumoniae* flavodoxin in coupling the reducing power of pyruvate:flavodoxin oxidoreductase to nitrogenase. Ferredoxins from aerobic, anaerobic, and photosynthetic nitrogen-fixing organisms failed to couple reducing power of pyruvate:flavodoxin oxidoreductase to nitrogenase. Nonphysiological low potential electron carriers like benzyl viologen and methyl viologen also were ineffective. Ferredoxins, benzyl viologen, and methyl viologen, reduced...
enzymatically, have been shown to donate electrons to nitrogenase (23–25). These results indicate that the reducing power of pyruvate:flavodoxin oxidoreductase can be coupled to nitrogenase only by flavodoxins. In contrast, flavodoxin, ferredoxins, benzyl viologen, and methyl viologen have been reported as electron acceptors for pyruvate:flavodoxin oxidoreductase of *E. coli* (21). Nonspecificity for electron acceptor was also reported for pyruvate:ferredoxin oxidoreductase of *C. acidi-urici* (13) and *Anaabaena cylindrica* (26).

Because *A. vinelandii* flavodoxin was only one-third as effective as *K. pneumoniae* flavodoxin in transferring electrons from pyruvate:flavodoxin oxidoreductase to *K. pneumoniae* nitrogenase, we examined its effectiveness in coupling electron flow to *A. vinelandii* nitrogenase (Table IV). It can be seen from these data that *A. vinelandii* flavodoxin was less effective than *K. pneumoniae* flavodoxin in coupling the reducing power of pyruvate:flavodoxin oxidoreductase to *A. vinelandii* and *K. pneumoniae* nitrogenase. On the other hand, *K. pneumoniae* flavodoxin was equally effective in transferring electrons to both nitrogenases. From these results, it seems that the interaction of pyruvate:flavodoxin oxidoreductase with flavodoxins rather than the interaction of flavodoxins with nitrogenase, dictates the observed activity. It will be interesting to examine the effectiveness of flavodoxins from other organisms, like *E. coli*, in this system. Yoch (4) reported that *E. coli* flavodoxin transferred electrons from illuminated chloroplasts to the nitrogenase of *K. pneumoniae* much more effectively than to the nitrogenase of *A. vinelandii*. *E. coli* pyruvate:flavodoxin oxidoreductase reduced various low potential electron acceptors, but all except flavodoxin were unable to mediate the activation of pyruvate formate lyase (21).

The change in flavodoxin spectra from the quinone to the hydroquinone brought about by pyruvate:flavodoxin oxidoreductase is illustrated by absorbance changes in Fig. 2. Nitrogenase then can change the hydroquinone to the semiquinone. These spectral changes demonstrate that pyruvate:flavodoxin oxidoreductase reduces flavodoxin to the hydroquinone state, which gets oxidized to semiquinone by transferring electrons to nitrogenase. Oxidation of chemically reduced *Azotobacter chroococcum* flavodoxin hydroquinone to semiquinone by nitrogenase was demonstrated by Yates (27). The data presented here demonstrate physiological reduction of flavodoxin by pyruvate:flavodoxin oxidoreductase and its oxidation by nitrogenase.

The stoichiometry of the pyruvate:flavodoxin oxidoreductase-coupled nitrogen fixation was deduced by determining all products of the reaction as mentioned under "Methods." The assays were carried out with varying amounts of pyruvate:flavodoxin oxidoreductase. The results of representative experiments (Table V) show that the reaction yields 1 mol each of ethylene, acetyl-CoA, and CO₂. These results demonstrate that oxidation of 1 mol of pyruvate:flavodoxin oxidoreductase transfers 2 electrons to flavodoxin which in turn transfers electrons to nitrogenase. Similar stoichiometric studies of the pyruvate:ferredoxin oxidoreductase of *C. acidi-urici* (13) demonstrated transfer of 2 electrons to benzyl viologen or *C. acidi-urici* ferredoxin. Experiments carried out with pyruvate:flavodoxin oxidoreductase, inactivated by exposure to air, failed to generate any of the reaction products.

**TABLE III**

| Electron carrier (μM) | Ethylene formed nmol |
|-----------------------|----------------------|
| *K. pneumoniae* flavodoxin (2.5, 5.9, 11.8) | 103, 204, 311 |
| *A. vinelandii* flavodoxin (3.0, 6.1, 30.5) | 30, 68, 287 |
| Clostridium pasteurianum ferredoxin (1.8, 3.6) | 0.0 |
| Rhodospirillum rubrum ferredoxin I (0.7, 1.4) | 0.0 |
| R. rubrum ferredoxin II (2.9, 5.8) | 0.0 |
| *A. vinelandii* ferredoxin I (2.9, 5.8) | 0.0 |
| Methyl viologen (0.5, 5.0) | 0.0 |
| Benzyl viologen (0.5, 5.0) | 0.0 |

**TABLE IV**

| Source of flavodoxin (μM) | Source of nitrogenase components I and II | Ethylene formed nmol |
|---------------------------|-----------------------------------------|----------------------|
| *K. pneumoniae* (11.8)    | *K. pneumoniae*                          | 310                  |
| *K. pneumoniae* (11.8)    | *A. vinelandii*                          | 315                  |
| *A. vinelandii* (11.7)    | *A. vinelandii*                          | 120                  |
| *A. vinelandii* (11.7)    | *K. pneumoniae*                          | 115                  |

**TABLE V**

| Exp. | Ethylene formed nmol | Acetyl-CoA formed nmol | CO₂ formed nmol | Product ratio |
|------|----------------------|------------------------|-----------------|---------------|
| 1    | 253                  | 239                    | ND              | 0.98          |
| 2    | 211                  | ND*                    | 201             | 1.05          |

*Not determined.
buffer (pH 7.4) as with 25 mM Tris-HCl buffer (pH 7.4) in pyruvate:flavodoxin oxidoreductase (Table I) is not due to phosphate buffer containing 1 mM MgCl₂ after 2 days of storage. We observed better than 90% activity of purified pyruvate:flavodoxin oxidoreductase in 0.2 M potassium phosphate buffer (pH 7.4) as with 25 mM Tris-HCl buffer (pH 7.4) in the assays. We observed better than 90% activity of purified pyruvate:flavodoxin oxidoreductase (in 0.2 M potassium phosphate buffer containing 1 mM MgCl₂) after 2 days of storage in dry ice. The relatively low yield of our preparations of pyruvate:flavodoxin oxidoreductase (Table I) is not due to inactivation of the enzyme; rather, the less active fractions containing the other iron-sulfur protein were discarded after each step of purification.

Extracts of NifJ⁻, but not of NifF⁻ mutant strains, can be activated (6-8, 28) in vitro by the iron-molybdenum cofactor (FeMo-co). It is possible that the nifJ gene product may be involved, directly or indirectly, in the biosynthesis of the iron-molybdenum cofactor. It is possible that the low potential iron-sulfur center of pyruvate:flavodoxin oxidoreductase may be involved in some reductive step of FeMo-co biosynthesis. Further investigations with NifJ⁻ mutants and the state of FeMo-co in these mutants should answer this question.

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