N₂ Fixation by *Streptomyces thermoaoutotrophicus* Involves a Molybdenum-Dinitrogenase and a Manganese-Superoxide Oxidoreductase That Couple N₂ Reduction to the Oxidation of Superoxide Produced from O₂ by a Molybdenum-CO Dehydrogenase

Markus Ribbe, Dilip Gadkari, and Ortwin Meyer

From the Lehrstuhl für Mikrobiologie, Universität Bayreuth, D-95440 Bayreuth, Germany

**EXPERIMENTAL PROCEDURES**

**Bacterial Strain and Growth Conditions—** *S. thermoaoutotrophicus* UBT1 (DSM 41605, ATCC 49746) was employed throughout this study (3). Bacteria were grown chemolithoautotrophically with CO as a sole source of carbon and energy in mineral medium supplied with trace elements (8) and 1.5 g of NH₄Cl/liter (8) at 65 °C. Fermentors of 70 liters total volume were flushed with a gas mixture composed of (v/v) 78% air, 13% CO, and 9% CO₂ at a flow rate of 3 liters/min. Bacteria were harvested by centrifugation and stored at −20 °C until use.

**Preparation of Subcellular Fractions—**Crude extracts were prepared by passing bacterial suspensions (200 g of wet weight in 200 ml of 50 mM potassium phosphate buffer, pH 7.5, containing 0.5 mM phenylmethylsulfonyl fluoride and few crystals of DNase I) about 20 times through a French pressure cell under anoxic oroxic conditions. Solutions were made anoxic by evacuation, sparging with N₂, and the addition of sodium dithionite (100 mg/ml). Cytoplasmic fractions were obtained from crude extracts by ultracentrifugation.

**Purification of Nitrogenase Proteins—** Cytoplasmic fractions were loaded onto a DEAE-52 cellulose column (height, 10.6 cm; diameter, 6.2 cm; bed volume, 250 ml) and eluted with 250 ml of phosphate buffer (50 mM, pH 7.5), followed by a linear gradient of 0 to 1 M NaCl in phosphate buffer. Fractions were analyzed for nitrogenase activity. The St2 protein appeared after elution in 100–125 ml of phosphate buffer; the St1 protein eluted at 0.3 M NaCl.

**St1 protein** was then subjected to centrifugation in a linear gradient of 5 to 35% (w/v) sucrose in phosphate buffer for 18 h at 100,000 × g and 4 °C. The St1 protein appeared at 19% sucrose. Fractions with nitrogenase activity were pooled, applied to a Sephadex G-150 gel filtration column (height, 44 cm; diameter, 2.5 cm; bed volume, 250 ml) and eluted with 250 ml of phosphate buffer (50 mM, pH 7.5), followed by a linear gradient of 0 to 1 M NaCl in phosphate buffer. Fractions were analyzed for nitrogenase activity. The St2 protein appeared after elution in 100–125 ml of phosphate buffer; the St1 protein eluted at 0.3 M NaCl.

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The purity of native proteins was examined by PAGE (9) at different pH values. Isoelectric focusing was performed with commercially available Isogel agarose isoelectric focusing plates (FMC Bio-Products).

**Enzyme Assays—**Nitrogenase was assayed in serum-stoppered 37-ml Wheaton vials containing 2 ml of phosphate buffer supplied with 2.5 mM ATP, 5 mM MgCl₂, 25 mM Na₂SO₄, 0.1 mg of St2 and 0.8 mg of St1. The gas atmosphere was pure N₂ or pure helium. Assays were kept unstirred at 65 °C. Ammonium formation (10) was followed with time (for 2 h if not otherwise indicated) in samples withdrawn from the vials. Calculations of specific activities of nitrogenase were based on the total amount of protein (St1 plus St2) present in the assay.

Nitrogenase activity in the experiments of Table IV was assayed in Wheaton vials of 100 ml of total volume, containing 15 ml of phosphate buffer, 2.5 mM ATP, 5 mM MgCl₂, and 30 mg of the cytoplasmic fraction. To this basic assay the additions specified in Table IV were made. The

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† To whom correspondence should be addressed. Tel.: 49-921-552729; Fax: 49-921-552727; E-mail: ortwin.meyer@uni-bayreuth.de.

‡ Recipient of a stipend from the Freistaat Bayern (München).

§ To whom correspondence should be addressed. Tel.: 49-921-552729; Fax: 49-921-552727; E-mail: ortwin.meyer@uni-bayreuth.de.

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1 The abbreviations used are: St1, dinitrogenase of *S. thermoaoutotrophicus*; St2, superoxide oxidoreductase, functioning as dinitrogenase reductase; St13, CO dehydrogenase; INT, 1-phenyl-2-(4-iodophenyl)-3-(4-nitrophenyl)-2H-tetrazolium chloride; SOD, superoxide dismutase; PAGE, polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography.

2 The nitrogenase components of *S. thermoautotrophicus* system of H₂O₂, the inability to reduce ethine or ethene, and a low MgATP requirement. In addition, the subunit structure from the known nitrogenases.
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anoxic assays were amended with cyanide and diethioletritol (4 mg each) and O₂-free gases, and the vials were equipped with a central cylinder containing 50 mg of sodium dithionite in 1 ml of phosphate buffer to trap traces of potentially contaminating O₂.

CO dehydrogenase was assayed spectrophotometrically using 1-methoxyphenazine methosulfate and INT as the electron acceptor (11). Formation of superoxide was assayed by the reduction of p-nitro blue tetrazolium as described previously (12). Electron transfer from St1 to physiological or artificial electron acceptors (50 μM acceptor in phosphate buffer) was assayed spectrophotometrically. H₂O₂ was analyzed colorimetrically with the iron-sensitive dye xylol orange (13).

Measurements of ATP Hydrolysis—Hydrolysis of MgATP during the reduction of N₂ to ammonium as shown in Fig. 5 was examined in Wheaton vials containing 0.625 mM ATP, 1.25 mM MgCl₂, 6.25 mM Na₂S₂O₄, 6 mg of St1, and 0.75 mg of St2 in 8 ml of phosphate buffer under a gas atmosphere of pure N₂ at 65 °C. Analysis of ammonium was as described above for the conventional nitrogenase assay. ATP hydrolysis was analyzed by isocratic high pressure liquid chromatography (HPLC) on an anion exchange column (PT 2564 NUCLEOSIL, 100–10 SB, Macherey-Nagel, Duren, Germany) using 500 mM potassium phosphate buffer (pH 3.5) as a mobile phase at a flow rate of 1 ml/min. The column outflow was continuously analyzed in a diode array detector (Waters 991, Waters, Eschborn, Germany).

Determination of Metals, Acid-labile Sulfide, and Sulphuryde—Protein samples were wet-ashed with concentrated sulfuric acid and H₂O₂, and analyzed for molybdenum with the dithion method (14) and for iron with the bathophenanthrolinedisulfonic acid method (15). Manganese, as well as molybdenum and iron, were also analyzed by inductively coupled plasma mass spectroscopy. The methylene blue method (16) was employed for the determination of acid-labile sulfide. Sulphuryde groups of native or SDS-treated proteins were estimated with 5,5’-dithio-bis(2-nitrobenzoic acid (17).

Miscellaneous Methods—Analysis of protein followed conventional methods (18, 19). N-terminal sequencing was by automated Edman degradation in a pulsed liquid-phase protein sequencer (Applied Biosystems) (20). Molecular weight determinations by gel filtration were on Superdex 200 prep grade (Pharmacia). Denaturant-free gels were carried out with DucoGelIV (MWG, Ebersberg) in combination with RFLPscan software (Scananalytic, Billerica). Polyacrylamide gels were calibrated with commercially available kits: ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa), bovine serum albumin (67 kDa), chymotrypsinogen A (25 kDa) (Pharmacia), and a protein ladder ranging from 10 to 120 kDa (Life Technologies, Inc.). H₂O₂ was in the experiment shown in Fig. 4 was analyzed by HgO to mercury conversion in a trace analytical RGD2 reduction gas detector (Technation, Düsseldorf) after gas chromatographic separation on molecular sieve (5 Å).

RESULTS

Purification and Subunit Composition of Nitrogenase Components—Centrifugation of cytoplasmic fractions of CO grown S. thermoautotrophicus revealed three distinct protein bands of brown color with approxiamtely molecular masses of 115, 49, and 147 kDa (Fig. 1) and the presence of the polypeptides St1, protein St2, and protein St3, respectively. The St3 protein was identified as CO dehydrogenase on the basis of the immeasurable molecular masses of 115, 49, and 147 kDa (sucrose density gradient centrifugation) or 41.5 kDa (gel filtration). Nodularin revealed subunits of 93, 33, and 18 kDa, designated L, M, and S, respectively (Fig. 1). They were noncovalently bound and occurred in a molar ratio of 1(L):0.8(M):0.9(S), suggesting a heterogeneous subunit structure.

The N termini of the St1 polypeptides had the sequences: ALPQTELRMGKPIRKXD (St1-L), MPNFAKYPEA-VIDEAVRLAEGYDGVK (St1-M), and MKRKYKNGTLYEADV (St1-S). The N termini of St1-L and the dinitrogenase β-subunit of Anabaena 7120 or Klebsiella pneumoniae showed 42.1 or 44.4% sequence similarity and 26.3 or 16.7% identity, respectively. The N termini of St1-M and the dinitrogenase α-subunit of Anabaena 7120 or K. pneumoniae showed 55.2 or 52.4% sequence similarity and 38.1 or 20.7% identity, respectively. The N terminus of St1-S revealed no significant sequence similarity to the α- or β-subunits of other nitrogenases or to the δ-subunit of alternative dinitrogenases.

Upon non-denaturating PAGE, the St2 protein revealed a single 40-kDa band at pH 6.5 or pH 7.5 (Fig. 1) or a single 98-kDa band at pH 8.5. Other methods revealed masses of 49 kDa (sucrose density gradient centrifugation) or 41.5 kDa (gel filtration). Denaturating PAGE showed a single noncovalently bound 24-kDa subunit, designated D (Fig. 1), suggesting a homodimeric subunit structure. The N terminus of the St2 polypeptide revealed no significant sequence similarity to sequenced nitorgenase polypeptides. Instead, St2-D showed 96.0% sequence similarity and 72.0% identity to the N termini of the manganese-containing SODs of Bacillus stearothermophilus (22) or Bacillus caldofex (23). In assays containing xanthine oxidase, xanthine, and O₂ for the production of peroxide anion radicals (O₂⁻) the St2 protein revealed O₂⁻ oxidizing activity of 126 units/mg of protein (1 unit equals 50% inhibition of NBT reduction). The reaction was insensitive to 3.5 mM potassium cyanide, 60 mM sodium azide, or 10 mM H₂O₂. Although the St2 protein displays many properties similar to a manganese SOD (24) it actually functions as a superoxide oxidoreductase transferring the electrons to the St1 protein (see below). In the presence of CO and O₂, CO dehydrogenase produced H₂O₂ and O₂⁻. The addition of commercial E. coli SOD effected an approximately 15% increase in apparent molecular mass of 129 kDa (Fig. 1). Denaturating PAGE revealed subunits of 93, 33, and 18 kDa, designated L, M, and S, respectively (Fig. 1). They were noncovalently bound and occurred in a molar ratio of 1(L):0.8(M):0.9(S), suggesting a heterogeneous subunit structure.

FIG. 1. Analysis of the components of the N₂ fixing system of S. thermoautotrophicus by native and denaturing PAGE. Lanes A–D, native PAGE; lanes E–H, denaturing PAGE. St1 protein (lanes B and F), St2 protein (lanes C and G), St3 protein (lanes D and H), reference proteins (lanes A and E); numbers are in kDa.

2 C. Schmidt, D. Gadkari, and O. Meyer, unpublished data.
to H2O2. Dithionite reduced St2 protein cannot replace MgATP in the reduction reaction of N2 or H.

MgATP, N2, and dithionite (Table III, lines 1 and 5–13). The proteinaceous nature of the nitrogenase is demonstrated by the lability to boiling or proteases (Table III, lines 2–4). MgADP could transfer 2.3 electrons to oxidized phenazine methosulfate with an activity of 77 nmol/mg of protein/min. Another suitable electron acceptor was 2,6-dichlorophenolindophenol (112 nmol/mg of protein/min), whereas viologen dyes, methylene blue, ferricyanide, INT, nitro blue tetrazolium, NAD(P), and riboflavin were ineffective.

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Ultraviolet and Visible Spectra, Contents of Metals, and Acid-labile Sulfide—The UV-visible spectrum of air-oxidized St1 protein revealed a protein peak at 274 nm and a broad nondistinct band in the region from 400 to 800 nm (Fig. 2). The dithionite-reduced spectrum was relatively featureless (Fig. 2). The visible spectra of oxidized and reduced St1 protein were similar to those of Kp1 (25) and different from those of Cp1 (26) or AvI (27). St1 is a molybdo-iron-sulfur protein which contains molybdenum, iron, and acid-labile sulfide in a 1:17:11 molar ratio (Table II).

The St2 protein exhibited a pale yellow-greenish color and contained substoichiometric amounts of manganese and zinc (Table II). Other components could not be demonstrated. In its overall shape the UV-visible spectrum of St2 was similar to that reported for the "SOD active" protein P1 from Euglena gracilis (28). The spectrum revealed a protein absorption at 276 nm and a broad absorption extending from 310 to 700 nm with a small maximum at 411 nm and a broad shoulder around 474 nm.

Reactions Catalyzed by the Proteins St1 and St2—Table III summarizes the requirements of the nitrogenase system for various components. The complete reaction mix for the in vitro reduction of N2 to NH4+ by nitrogenase required St1, St2, MgATP, N2, and dithionite (Table III, lines 1 and 5–13). The proteinaceous nature of the nitrogenase is demonstrated by the lability to boiling or proteases (Table III, lines 2–4). MgADP cannot replace MgATP in the reduction reaction of N2 or H+ (Table III, line 14). Table III also demonstrates that in the complete nitrogenase assay CO dehydrogenase cannot replace St1, St2, or both for the reduction of N2 or H+ (lines 15–17).

Maximum activity for the formation of NH4+ from N2 was obtained at a molar ratio of St1 to St2 of 2.2:1 (Fig. 3). An ATP-regenerating system (e.g. creatine phosphate and creatine kinase) was not required. A 10-fold excess of MgADP over MgATP had no effect on nitrogenase activity. MgADP is apparently not inhibitory for S. thermoautotrophicus nitrogenase. Formation of NH4+ from N2 as well as formation of H2 from H+ was linear with time for at least 2.5 h (Fig. 4, 5). In the absence of St2, only H2 but no ammonium was formed (Fig. 4). Reduction of ethene or ethylene by mixtures of St1 and St2 was below the detection limit of 0.2 pmol of carbon of the gas chromatographic assay applied. There was a linear correlation between MgATP hydrolysis to MgADP and NH4+ formation from N2 (Fig. 5). At the assay temperature of 65 °C, ATP was almost chemically stable, whereas significant hydrolysis of ADP to AMP and inorganic phosphate occurred (Fig. 5). There was a linear correlation between the molar formation of ammonium from N2 and the molar amount of MgATP under different assay conditions (Fig. 6). The regression line followed the equation [NH4+]/[ATP] = 0.35833 × [ATP] + 0.0409. Formation of 2 mol of NH4+ is equivalent to the reduction of 1 mol of N2, had a mean correlation between the molar formation of ammonium from N2 and the molar amount of MgATP under different assay conditions (Fig. 6). The regression line followed the equation [NH4+] = 0.35833 × [ATP] + 0.0409. Formation of 2 mol of NH4+ is equivalent to the reduction of 1 mol of N2, had a mean requirement of 5.5 ± 1.0 mol of MgATP (n = 22). The minimum and maximum MgATP/N2 ratios were 3.85 or 11.34, respectively (Fig. 6). This is significantly less than the minimum of 16 ATP reported for nitrogenases from other sources (29–31).

In assays with limiting amounts of N2 in helium, 1 mol of N2 was reduced yielding 2.2 ± 0.2 mol of NH4+ and 0.9 ± 0.2 mol...
of H₂. The data would support the following equation for the reduction of N₂ by the proteins St1 and St2

\[ \text{N}_2 + 4-12\text{MgATP} + 8\text{H}^+ + 8e^- \rightarrow 2\text{NH}_3 + \text{H}_2 + 4-12\text{MgADP} + 4-12\text{Pi} \]  

(Eq. 1)

**N₂ Fixation Is Linked to the Oxidation of Superoxide**—The CO dehydrogenases from *Oligotropha carboxidovorans* and *S. thermoautotrophicus* can transfer the electrons formed upon CO oxidation (CO + H₂O → CO₂ + 2e⁻ + 2H⁻) to O₂, thereby producing superoxide anion radicals (O₂⁻) and hydrogen peroxide (H₂O₂). In assays containing purified CO dehydrogenase, St1 and St2 protein, and MgATP under an atmosphere of (v/v) 50% CO and 50% air, N₂ was reduced to ammonium, indicating that N₂ reduction was coupled to the oxidation of CO. Ammonium was not formed in the absence of CO. The requirement of O₂ for N₂ reduction in cytoplasmic fractions (Table IV) is explained by the formation of O₂⁻ by CO dehydrogenase and the subsequent reoxidation of O₂⁻ to O₂ by the St2 protein. The functioning of O₂⁻ as an electron donor for N₂ reduction is further substantiated by the sensitivity of the reaction to added SODs, the ability to replace CO dehydrogenase for xanthine oxidase, plus a suitable substrate or to provide O₂⁻ chemically from riboflavin plus light (Table IV). MnSODs from *Escherichia coli* and *B. stearothermophilus* could not substitute for the St2 protein (Table IV). Oxidation of H₂ by CO dehydrogenase in the presence of O₂ also leads to O₂⁻ formation (Table IV).

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**TABLE II**

Contents of metals, acid-labile sulfide, and sulfhydryls of St1 and St2 protein from *S. thermoautotrophicus*

| Protein | Mo | Fe | Zn | Mn | V | Acid-labile sulfide | Sulfhydryls Available<sup>a</sup> | Total<sup>b</sup> |
|---------|----|----|----|----|---|---------------------|------------------------|---------|
| St1     | 0.7 ± 0.1 | 12 ± 1 | 3.4 | ND | <0.06 | 8 ± 1 | 4 ± 0.1 | 12 ± 1 |
| St2     | <0.04 | <0.07 | 0.1 | 0.24 ± 0.03 | <0.02 | 0.02 | 0 | 0 |

Molecular masses of 144 kDa (St1) and 48 kDa (St2) have been used for calculations. The values of acid-labile sulfide, molybdenum, and iron are the mean of six, manganese the mean of two and the sulfhydryls the mean of four independent determinations.

<sup>a</sup> Number of sulfhydryl groups available for 5,5'-dithio-bis-2-nitrobenzoic acid at the surface of native protein.

<sup>b</sup> Total number of sulfhydryl groups of denaturated protein.

<sup>c</sup> Not determined.

**TABLE III**

Requirements of nitrogenase for the reduction of N₂ or H⁺

| Line | Assay components | Activities | N₂ reduction<sup>a</sup> | Proton reduction<sup>b</sup> |
|------|------------------|------------|--------------------------|-----------------------------|
| 1    | + + + + + + + +  | 106        | 61                       |                             |
| 2    | + + + + + + + +  | 0          | 0                        |                             |
| 3    | + + + + + + + +  | 0          | 0                        |                             |
| 4    | + + + + + + + +  | 0          | 0                        |                             |
| 5    | + + + + + + + +  | 0          | 0                        |                             |
| 6    | + + + + + + + +  | 0          | 0                        |                             |
| 7    | + + + + + + + +  | 0          | 0                        |                             |
| 8    | + + + + + + + +  | 0          | 0                        |                             |
| 9    | + + + + + + + +  | 0          | 0                        |                             |
| 10   | + + + + + + + +  | 0          | 0                        |                             |
| 11   | + + + + + + + +  | 0          | 0                        |                             |
| 12   | + + + + + + + +  | 0          | 0                        |                             |
| 13   | + + + + + + + +  | 0          | 0                        |                             |
| 14   | + + + + + + + +  | 0          | 0                        |                             |
| 15   | + + + + + + + +  | 0          | 0                        |                             |
| 16   | + + + + + + + +  | 0          | 0                        |                             |
| 17   | + + + + + + + +  | 0          | 0                        |                             |

<sup>a</sup> N₂ reduction is in nmol NH₄⁺/mg protein/h.

<sup>b</sup> Proton reduction is in nmol H₂/mg protein/h.

<sup>c</sup> St1 and St2 were boiled for 10 min.

<sup>d</sup> St1 and St2 (1 mg each) were preincubated for 16 h at 20 °C with protease K (12.5 μg) and then assayed for activity.

<sup>e</sup> Same as<sup>d</sup>, except that protease K was replaced by trypsin (50 μg).

<sup>f</sup> Same results were obtained with 3 or 30 mg of ADP per assay.

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<sup>3</sup> A. Oberfrank and O. Meyer, unpublished data.
During chemolithoautotrophic utilization of H₂, *S. thermoautotrophicus* employs a membrane-bound hydrogenase for energy generation; this hydrogenase cannot produce O₂. Under these conditions the hydrogenase activity of CO dehydrogenase (32, 33) is used for the generation of O₂ as an electron donor for N₂ fixation.

**Effect of O₂ and H₂O₂ on the Catalytic Activity of Proteins St1 and St2**—When cytoplasmic fractions of *S. thermoautotrophicus* were kept for 24 h at room temperature under N₂, air, or O₂, nitrogenase activities ranged from 20.2 to 29.6 nmol of NH₄⁺/mg of protein/h. Shaking of the purified proteins St1 and St2 for 24 h in air also did not affect nitrogenase activity. Under oxic conditions St1 was purified 11-fold with a yield of 1% and a specific activity of 139 nmol of NH₄⁺/mg of protein/h, and St2 was purified 21-fold, with a yield of 31% and a specific activity of 233 nmol of NH₄⁺/mg of protein/h. The results characterize the N₂-fixing system of *S. thermoautotrophicus* as absolutely O₂-insensitive.

The insensitivity of St2 to H₂O₂ has already been mentioned.

**TABLE IV**

| Conditions | Nitrogenase activity\(^a\) |
|------------|---------------------------|
| CO plus air\(^b\) | 22.6 |
| CO plus air plus SOD (E. coli or B. stearothermophilus) \(^c\) | 0.0 |
| H₂ plus air \(^d\) | 25.2 |
| Xanthine oxidase plus xanthine plus air \(^e\) | 35.3 |
| Xanthine oxidase plus hypoxanthine plus air \(^e\) | 32.1 |
| Riboflavin plus light plus air \(^\beta\) | 20.6 |
| CO plus N₂ \(^\gamma\) | 0.0 |
| CO plus N₂, O₂ was added after 3 h \(^h\) | 33.2 |

\(^a\) Specific activities are in nmol NH₄⁺/mg of cytoplasmic protein/h. For basic assay conditions refer to "Experimental Procedures."  
\(^b\) Basic assays were sparged with (vol/vol) 50 CO plus 50 air.  
\(^c\) Basic assays were supplied with SOD from *E. coli* (0.45 mg) or *B. stearothermophilus* (0.15 mg) and sparged with (vol/vol) 50% CO plus 50% air.  
\(^d\) Basic assays were sparged with (vol/vol) 50% H₂ plus 50% air.  
\(^e\) Basic assays were supplied with bovine milk xanthine oxidase (6.4 mg), xanthine (0.7 mM), or hypoxanthine (0.7 mM).  
\(^\beta\) Basic assays containing 10 mM riboflavin were sparged with air and illuminated with light (50 W/m²).  
\(^\gamma\) Basic assays were sparged with (vol/vol) 50% CO plus 50% N₂ under anoxic conditions.  
\(^\beta\) Basic assays were sparged for 3 h with (vol/vol) 50% CO plus 50% N₂ under anoxic conditions, and after 3 h N₂ was replaced by air.
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Since $H_2O_2$ is another product in the formation of $O_2$ by CO dehydrogenase or xanthine oxidase, the results of Table IV also indicate that the entire nitrogenase reaction in subcellular fractions of S. thermoautotrophicus is insensitive to $H_2O_2$.

**DISCUSSION**

$N_2$ fixation by S. thermoautotrophicus involves CO dehydrogenase, free $O_2$, and $O_2^\cdot$ as electron carriers and the proteins St2 and St1 (Fig. 7). CO dehydrogenase generates superoxide anion radicals ($O_2^\cdot$) from $O_2$, which makes $N_2$ fixation in S. thermoautotrophicus obligately $O_2^\cdot$-dependent and establishes a molecular coupling via $O_2^\cdot$ which is in contrast to the electronic coupling in the known nitrogenase systems. The $O_2^\cdot$ anions are free intermediates and can be trapped by SOD (Table IV). Consequently, interaction of CO dehydrogenase and the St2 protein for $O_2^\cdot$ transfer is not required. Generally, $O_2^\cdot$ is considered a highly reactive and destructive metabolic by-product that requires detoxification, e.g. by superoxide dismutases. $N_2$ fixation in S. thermoautotrophicus shows that the redox couple $O_2/O_2^\cdot$ (E’$\theta$ = −160 mV) operates as an electron carrier with similar efficiency as ferredoxins, flavodoxins, or hydroquinones in the known nitrogenase systems (34, 35). In addition, the use of $O_2^\cdot$ in $N_2$ fixation by S. thermoautotrophicus seems to be a powerful mechanism to scavenge $O_2^\cdot$ radicals.

The dinitrogenase reductases known so far are $\gamma_2$ dimeric iron proteins with molecular mass = 63 kDa and contain 4Fe and 4S$^{2-}$ atoms per dimer (36). In contrast, the St2 protein of S. thermoautotrophicus has been identified as a manganese-superoxide oxidoreductase with molecular mass = 48 kDa and does not contain Fe or S$^{2-}$ (Table II). In contrast to SOD, St2 is unable to disproportionate $O_2^\cdot$ into $O_2$ and $H_2O_2$. The function of St2 is rather to generate electrons through the reoxidation of $O_2^\cdot$ ions to $O_2$ and to deliver them to the St1 protein (Fig. 7). Like the known nitrogenases, the S. thermoautotrophicus system also has a requirement for MgATP. With S. thermoautotrophicus nitrogenase the most efficient MgATP/$N_2$ ratio is 4, instead of 16 reported for nitrogenases from other sources, indicating its superb efficiency. Future work must unravel how MgATP interacts with the nitrogenase proteins.

It is likely that the reduction of $N_2$ takes place at the St1 protein of S. thermoautotrophicus. The known MoFeS-dinitrogenases are complex $\alpha_2\beta_2$ tetrameric proteins with $M_w$ = 230 kDa containing 2 molybdenum atoms and 30–34 iron atoms and an approximately equivalent number of acid-labile sulfide atoms (36). The St1 protein was also identified as a MoFeS-protein but with molecular mass = 144 kDa and a differing $\alpha_2$ tetrameric subunit structure (Figs. 1 and 7). It revealed per molybdenum atom, 13.8–21.7 iron atoms and 8.8–15 acid-labile sulfide atoms (Table II). A substoichiometric amount of acid-labile sulfide compared with iron is frequently found with nitrogenases (37). There was a moderate sequence similarity between the N-terminal sequences of the subunits St1-L and Kp1-$\beta$ or St1-M and Kp1-$\alpha$, although we are aware of the limitations of a comparison of subunit N termini. The overall reaction catalyzed by S. thermoautotrophicus nitrogenase compares to that of known nitrogenases, including the concomitant formation of $H_2$ (Fig. 4).

In consequence of the inability of the S. thermoautotrophicus nitrogenase to reduce ethine or ethene, the widely used ethine reduction assay is not applicable for measuring nitrogenase activity in environmental samples inhabited by bacteria performing this new type of $N_2$ fixation. It can be expected that the insensitivity of the S. thermoautotrophicus nitrogenase to $O_2$ will open new avenues for practical applications. Studies on the electron paramagnetic resonance properties of the metal centers in the S. thermoautotrophicus nitrogenase system as well as on the genetics and molecular biology of CO dehydrogenase (St3 protein) and St1 and St2 proteins, which are currently underway, will add further clues to the understanding of the novel nitrogenase system.

**REFERENCES**

1. Meyer, O., Meyer, W., Gadkari, D., Zellmann, H., Schricker, K., and Schmitt, M. (1991) in The Third Symposium on Bio/Technology of Coal and Coal-derived Substances (Rehm, H. J., Fakoussa, R. M., Schacht, S., and Klein, J., eds) pp. 111–121, DMT-Institute for Applied Environmental Chemistry, Essen
2. Meyer, O. (1997) Kohlererei im Fichtelgebirge, Frankenwald und Bayerischen Wald. pp. 1–168, Erich Goltze, Göttingen
3. Gadkari, D., Schricker, K., Acker, G., Kroppenstedt, R. M., and Meyer, O. (1990) Appl. Environ. Microbiol. 56, 3727–3734
4. Gadkari, D., Morosdorf, G., and Meyer, O. (1995) J. Bacteriol. 174, 6840–6843
5. Gadkari, D., and Meyer, O. (1994) Biotechnology. 2, 27
6. Ribbe, M., Gadkari, D., and Meyer, O. (1994) Bioengineering 2, 80
7. Dalton, H., and Mortenson, L. E. (1972) Biochemistry 21, 231–260
8. Meyer, O., and Schlegel, H. G. (1983) Annu. Rev. Microbiol. 37, 277–310
9. Laemmlı, U. K. (1970) Nature 272, 680–685
10. Gadkari, D. (1984) Zentralbl. Mikrobiol. 139, 623–631
11. Kraut, M., Hugendieck, I., Herwig, S., and Meyer, O. (1989) Arch. Microbiol. 152, 335–341
12. Beauchamp, C., and Fridovich, I. (1971) Anal. Biochem. 44, 276–287
13. Wolf, S. P. (1994) Methods Enzymol. 233, 182–189
14. Cardenas, J., and Mortensen, L. E. (1974) Anal. Biochem. 60, 372–381
15. Schade, A. L., Noma, Y., Reinhart, R. W., and Miller, J. R. (1954) Proc. Soc. Exp. Biol. Med. 87, 443–449
16. Meyer, O. (1982) J. Biol. Chem. 257, 1333–1341
17. Habeeb, A. F. S. A. (1972) Methods Enzymol. 25B, 457–464
18. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
19. Schmidt, K., Lianen-Jensen, S., and Schlegel, H. G. (1963) Arch. Microbiol.
N\textsubscript{2} Fixation by S. thermoautotrophicus

20. Löw, A., Sprinzl, M., and Faulhammer, H. G. (1993) Eur. J. Biochem. 215, 473–479
21. Meyer, O., Frunzke, K., Tachil, J., and Volk, M. (1993) in Molybdenum Enzymes, Cofactors, and Model Systems (Stiefel, E. I., Coucouvanis, D., and Newton, W. E., eds) pp. 50–68, American Chemical Society, Washington, D. C.
22. Bowler, C., Van Kaer, L., Van Camp, W., Van Montagu, M., Inze, D., and Dhaese, P. (1990) J. Bacteriol. 172, 1539–1546
23. Chambers, S. P., Brehm, J. K., Michael, N. P., Atkinson, T., and Minton, N. P. (1992) FEBS Lett. 91, 277–284
24. Misra, H. P., and Fridovich, I. (1971) Arch. Biochem. Biophys. 489, 317–322
25. Eady, R. R., Smith, B. E., Cook, K. A., and Postgate, J. R. (1972) Biochem. J. 128, 655–675
26. Dalton, H., Morris, J. A., Ward, M. A., and Mortenson, L. E. (1971) Biochemistry 10, 2066–2072
27. Shah, V. K., and Brill, W. J. (1975) Biochim. Biophys. Acta 305, 445–454
28. Lengfelder, E., and Elstner, E. F. (1979) Z. Naturforsch. 34c, 374–380
29. Burris, R. H. (1991) J. Biol. Chem. 266, 9339–9342
30. Dean, D. R., Bolin, J. T., and Zheng, L. (1993) J. Bacteriol. 175, 6737–6744
31. Kim, J., Woo, D., and Rees, D. C. (1993) Biochemistry 32, 7104–7115
32. Meyer, O., Frunzke, K., Gadkari, D., Jacobitz, S., Hugendieck, I., and Kraut, M. (1990) FEBS Lett. 87, 253–260
33. Santiago, B., and Meyer, O. (1996) FEBS Lett. 136, 157–162
34. Yates, M. G. (1972) FEBS Lett. 27, 63–67
35. Evans, H. J., and Burris, R. H. (1992) in Biological Nitrogen Fixation (Stacey, G., Burris, R. H., and Evans, H. J., eds) pp. 1–42, Chapman & Hall, New York
36. Eady, R. R. (1996) Chem. Rev. 96, 3013–3030
37. Blanchard, C. Z., and Hales, B. J. (1996) Biochemistry 35, 472–478