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CALCIUM IS REQUIRED FOR THE REDUCTION OF SULFITE FROM HYDROGEN IN A RECONSTITUTED ELECTRON TRANSFER CHAIN FROM THE SULFATE REDUCING BACTERIUM, Desulfovibrio gigas

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SUMMARY Calcium is found a strong stimulator of sulfite reduction from hydrogen. A coupling protein of molecular weight 65,000 can be isolated from Desulfovibrio gigas. It functions in a reconstituted electron transfer chain between hydrogenase and sulfite reductase. Its N-terminal sequence shows high homologies with calcium or magnesium binding sites from other calcium-binding proteins.

Although it has been known for many years that ferredoxin (1) or flavodoxin (2) can couple the reduction of sulfite to hydrogen sulfide in extracts of Desulfovibrio gigas, a complete electron transfer chain from hydrogenase (3) to desulfoviridin, the dissimilatory bisulfite reductase (4), has never been described. Thus studies of this important reaction in the energetics of sulfate reducing bacteria have been limited to the use of the artificial electron carrier methyl viologen as a coupling agent between hydrogen and desulfoviridin. We want to report here that this coupling can be obtained by the physiological electron carrier cytochrome c_3 (5), ferredoxin or flavodoxin and a newly discovered protein, after addition of calcium ions to the system.

MATERIALS AND METHODS

Organism and growth conditions: the growth conditions of D. gigas (ATCC 19364) and preparation of cell-free extracts were described previously (6).

Enzyme activity and analytical procedures: a Warburg manometric assay was used for the determination of the enzymatic activities (4). Molecular weights were determined by 12% SDS gel electrophoresis (7). Protein standards were: lysozyme (MW=14,000),

Abbreviations used: PAGE: polyacrylamide gel electrophoresis; SDS: sodium dodecylsulfate; EDTA: ethylene diamine tetracetate; Tris: tris (hydroxymethyl) aminomethane; DEAE: diethyl aminoethyl.

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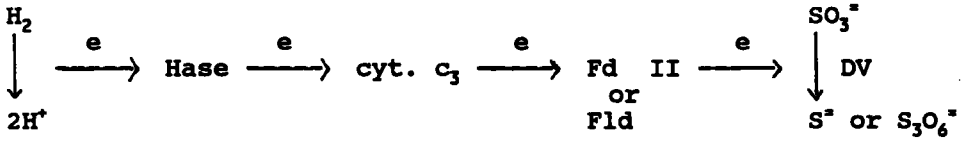
soybean trypsin inhibitor (MW=21,500), carbonic anhydrase (MW=31,000), ovalbumin (MW=45,000), bovine serum albumin (MW=66,200), and phospholipase b (MW=92,500). Protein concentrations were determined by the method of Bradford (8). Electroeluction was used for the final purification developed by Hunkapiller (9). PAGE native gel electrophoresis was as described by Davis (10). Metal content was determined by plasma emission spectroscopy, using the Jarrell-Ash model 750 atom comp. A Shimadzu spectrophotometer (UV 265) was used for absorption measurements. N-terminal sequence was determined by an Applied Biosystem sequencer (model 470A) using the method of Edman and Begg (11).

Protein purification: hydrogenase, desulfoviridin, cytochrome c_3, and ferredoxin II were purified as described previously (4,5,12,13). An extract devoid of cytochrome c_3, ferredoxin and flavodoxin was obtained as described previously by passing a D. gigas crude extract through a small DEAE column (6). Purification of the coupling protein (CP) was obtained from the dialyzed supernatant of a D. gigas crude extract treated with 30% ammonium sulfate. A pure protein, as judged by SDS electrophoresis, was obtained after several chromatographic steps including DEAE-cellulose, DEAE-Biogel, and electro-elution. The coupling efficiency of each fraction was checked by Warburg respirometry using the system described on figure 2.

RESULTS AND DISCUSSION

The electron transfer chain for sulfite reduction from molecular hydrogen can be described as follows (18):



(Hase: hydrogenase; cyt. c_3: cytochrome c_3; Fd II: ferredoxin II; Fld: flavodoxin; DV: desulfoviridin; e: electrons)

However, when pure proteins were used, no significant activity was found. When dialyzed crude extracts were utilized in this reaction, it was found that the rate was significantly increased after addition of either calcium or magnesium ions (figure 1). Addition of EDTA to such systems decreased the reaction rate (figure 1), however, the interpretation of the effects of this compound is difficult since the system contains non-heme iron which is easily chelatable. It was discovered that another factor was needed for the effect of calcium or magnesium. After purification, this factor was identified as a protein, thus named coupling protein (CP), the characteristics and properties of which are described below. The effects of the addition of the purified CP and calcium on a sulfite-reducing system containing hydrogenase, desulfoviridin, tetra-heme cytochrome c_3, and ferredoxin are shown on figure 2. The same stimulation of hydrogen uptake was obtained when ferredoxin was replaced by flavodoxin. When Ca^{++} and CP were absent, the reaction rate was but negligible. Thus, a complete electron transfer chain,

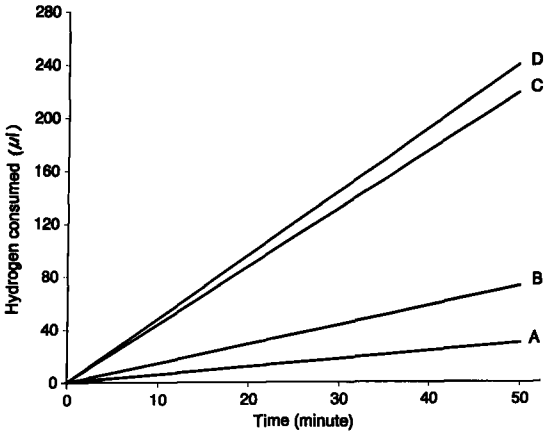


Figure 1. Effect of the addition of EDTA, Ca^{++}, and Mg^{++} on the reduction of sulfite from molecular hydrogen by a crude extract from D. gigas. Each Warburg flask contained: 30 µmole Na_2SO_3, 10 mg protein of crude extract, 150 µmoles Tris-maleate buffer (pH 6.5); $CdSO_4$ and NaOH were added in the center well. A: D.g. crude extract + 300 nmoles EDTA; B: D.g. crude extract - EDTA; C: D.g. crude extract + 40 µmoles Ca^{++}; D: D.g. crude extract + 40 µmoles Mg^{++}.

constituted of pure, soluble proteins, is functional in vitro, after addition of both CP and calcium ions, from the oxidation of molecular hydrogen to the reduction of sulfite.

After SDS gel electrophoresis, the molecular weight of CP was estimated to be 65,000. Results from plasma emission did not show any significant amount of metal in the dialyzed protein. However, after incubation of the protein with calcium and separation from the calcium containing buffer by centrifugation through a centricon membrane, CP was found to be able to bind 4 atoms of Ca^{++} per molecule. The

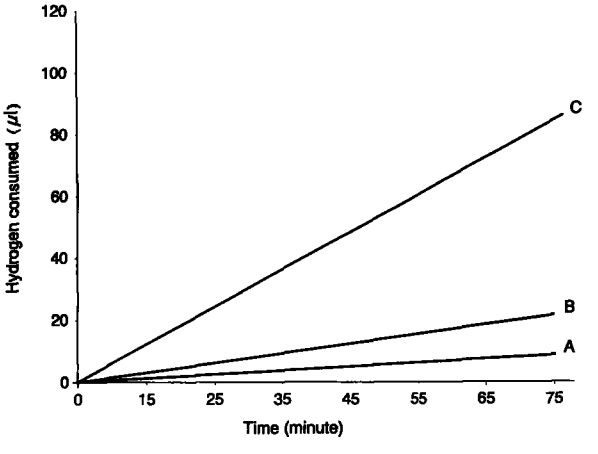


Figure 2. Effect of the coupling protein and calcium on the reduction of sulfite from molecular hydrogen by a reconstituted electron transfer chain. Each Warburg flask contained: 30 µmoles Na_2SO_3, 200 units D.g. hydrogenase, 20 nmoles D.g. cytochrome c_3, 40 nmoles D.g. ferredoxin II, 40 nmoles D.g. desulfoviridin, 150 µmole Tris-maleate buffer (pH 6.5); $CdSO_4$ and NaOH were added in the center well. A: without CP; B: 20 nmoles CP - Ca^{++}; C: 20 nmoles CP + 40 µmoles Ca^{++}.

analysis of the N-terminal part of CP is shown below together with the structure of the calcium binding sites of human calmodulin (14):

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1       5          10         15          20
A-T-L-K-L-A-M-D-A-D-P-E-T-L-D-P-()-V-E-L  D. gigas CP

              D-A-D-G-N-G-T-I-D-F--P-E
              D-K-D-G-D-G-T-I-T-T--K-E    Human calmodulin
              D-K-D-G-N-G-Y-I-S-A--A-E    calcium-binding sites
              D-I-D-G-D-G-Q-V-N-Y--E-E
```

(): unidentified residue

As expected from a protein containing an "EF-hand" (15) calcium binding site, the first 7 residues are very likely to form an α-helix. The peptide 8 to 19 has amino-acids with oxygen-containing side chains (Asp 8, 10, 15; Glu 12 and 19, and Thr 13) which are ordinarily found in the calcium binding loop. This peptide also contains two proline residues which can be visualized as replacing the two α-helix breakers glycine residues found in calmodulins. The last two residues of CP which have been sequenced so far, Glu 19 and Leu 20 are also compatible with the starting of the second α-helix of the "EF-hand" (15).

To our knowledge this preliminary work is the first report concerning the role of calcium and of a calcium-binding protein, in the stimulation of electron transfer in a bacterium. Further work remains to be done to fully characterize the coupling protein and define its mechanism of action. It is possible to speculate that it acts by stabilizing the complexes which have already been shown to exist between D. gigas cytochrome c_3, flavodoxin (16) and ferredoxin (17) and their interaction with hydrogenase and the terminal reductase. It also remains to be seen if this effect of calcium on an important reaction for energy conservation in Desulfovibrio species can be generalized to other prokaryotic or even eukaryotic systems.

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