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EVIDENCE FOR ONE-ELECTRON TRANSFER BY THE FE PROTEIN OF NITROGENASE

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SUMMARY

The number of electrons transferred per molecule of the Fe protein of nitrogenase from Clostridium pasteurianum was determined. The Fe protein was enzymically oxidized in the presence of MgATP and a small amount of MoFe protein, and dithionite was introduced to reduce part of the Fe protein. From the decrease in absorbance at 430 nm upon addition of dithionite and the amount of dithionite added, we conclude that one oxidized Fe protein molecule (dimer of 55,000 dalton) accepts one electron from dithionite. These calculations were based on our value of 6,600 $M^{-1}cm^{-1}$ for the extinction coefficient at 430 nm of the difference spectrum between oxidized and reduced Fe protein.

INTRODUCTION

The nitrogen fixing enzyme system, nitrogenase, consists of two proteins, the Fe protein and the MoFe protein (1,2). The sequence of electron transfer is: external electron donor —— Fe protein —— MoFe protein —— N_2. Iron-sulfur centers participate in this electron transfer, with the iron-sulfur center of the Fe protein exhibiting properties, such as EPR spectra, similar to those of the smaller ferredoxins; whereas, the MoFe protein is more complex and may contain iron-sulfur clusters of presently unknown structure.

There is considerable evidence in support of one 4Fe-4S cluster per Fe protein molecule of two subunits (2,3,4), and this is compatible with one-electron transfer

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Nevertheless, previously reported attempts to determine the number of electrons have suggested that the Fe protein tranfers a total of 2 electrons per protein molecule (5,6). These studies are complicated by the rapid O_2-inactivation of the Fe protein. We have presented a method for reversible, enzymic oxidation of active Fe protein (4) and have determined the extinction coefficient for the difference spectrum between reduced and enzymically-oxidized Fe protein (7). The present paper reports results from titrations of enzymically-oxidized Fe protein with standardized solutions of sodium dithionite and supports one-electron transfer by the Fe protein.

METHODS

The nitrogenase proteins were purified from cells of Clostridium pasteurianum W5 (7,8). Anaerobic techniques, analytical procedures, and the procedure for monitoring enzymic oxidation of the Fe protein were as previously described (4, and refs. therein). The cuvets (10 mm light path) for anaerobic spectrophotometry were closed with rubber vaccine stoppers and carefully evacuated and flushed with purified H_2. The last traces of O_2 were removed by the following procedure: Buffer or the reaction mixture for enzymic oxidation was injected followed by small volumes of 0.1 M $Na_2S_2O_4$, and the cuvets were shaken vigorously until there was no decrease in the dithionite absorbance at 315 nm upon further shaking, and the final excess of dithionite was ca 0.1 mM. The stock dithionite solution for titration was standardized by injection of 10 µl samples into 1.00 ml 50 mM tris, pH 8.0 in cuvets that had been subjected to the treatment for O_2 removal. The dithionite concentration was calculated from the absorbance increase at 315 nm (ε = 8,000 $M^{-1}cm^{-1}$; ref. 9). 10 µl volumes of stock dithionite solution were injected with the same Hamilton syringe for standardization, and for reduction of the Fe protein.

RESULTS AND DISCUSSION

Introduction of dithionite to enzymically oxidized Fe protein leads to rapid reduction and decrease in absorbance at 430 nm (Figure 1). Nevertheless, reduction is sufficiently slow for reoxidation to interfere. Because the rate of reoxidation is nearly constant for a considerable period of time, the electrons removed because of reoxidation can be corrected for by extrapolation back to the time of addition of dithionite. The amount of Fe protein reduced by the given amount of dithionite was then calculated from the absorbance decrease, corrected by extrapolation. We have previously determined an extinction coefficient of 6,600 $M^{-1}cm^{-1}$ (per 4Fe-4S cluster) for this absorbance change (7) by using similar enzymic oxidations and the MgATP-dependent reaction between the Fe protein and iron chelators

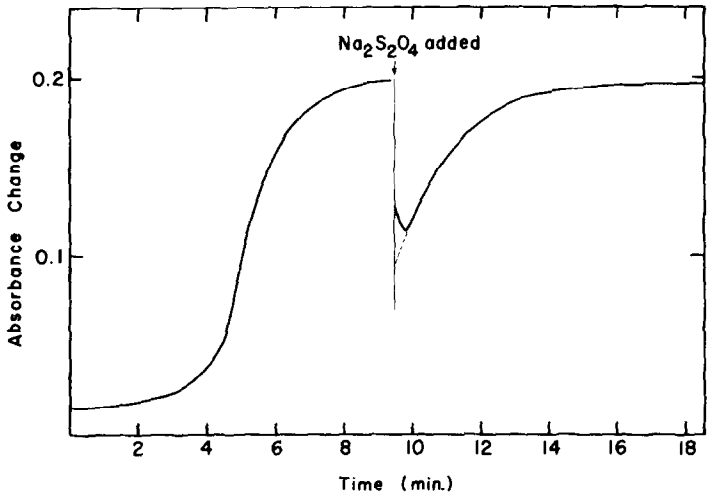

Figure 1. Absorbance changes at 430 nm during enzymic oxidation of the Fe protein followed by reduction with standardized dithionite. Reaction mixtures at 25°C contained in 1.00 ml 50 mM tris, pH 8.0: 2.5 mg Fe protein, 0.02 mg MoFe protein, 0.1 mg creatine kinase (from rabbit muscle; Sigma type 1), 1 μmol ATP, 5 μmol $MgCl_2$, 10 μmol creatine phosphate. 7.4 nmol $Na_2S_2O_4$ in 10 μl was introduced as indicated. Extrapolation to correct for reoxidation is indicated by the broken line.

(10). We have used bathophenanthroline disulfonate as chelator to determine the concentration of active (but not O_2-inactivated) Fe protein. Dithionite is a two-electron donor to nitrogenase (11), and the number of electrons added were calculated from the results of the standardization of the stock dithionite solution.

The data in Figure 1 indicate that the number of electrons accepted per Fe protein molecule was 0.97. Other experiments gave these values: 0.81; 0.89; 0.94; 1.16. Thus, these experiments strongly suggest that the Fe protein is a one-electron acceptor or donor.

The need to correct for reoxidation by extrapolation introduces some uncertainty in the interpretation of these experiments, but the correction introduced is rather small, and even without the extrapolation the calculated e^-/Fe protein ratios are much closer to 1 than to 2.

A major source of error is leakage of O_2 into the cuvets. This problem is largely circumvented, however, by our procedure for removal of traces of O_2 in the

cuvets, and by the use of closely identical conditions for standardization of dithionate solutions and for reduction of oxidized Fe protein. Replicate determinations during standardization were in good agreement, indicating that the problem of O_2-leakage had been minimized.

An advantage of our experimental design, compared with reports on dye-oxidation of reduced Fe protein (5) or dithionite-reduction of dye-oxidized Fe protein (6), is that in our experiments only active Fe protein becomes oxidized and can subsequently accept electrons from dithionite. Inactive Fe protein and other possible redox-active substances are reduced by the excess dithionite at the start of the experiment and remain reduced throughout. Moreover, only reactions that are compatible with active cell-free nitrogen fixation are employed, and the Fe protein is not exposed to chemicals that could introduce oxidation states other than those found in catalytically functional Fe protein.

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