Nitrogenase of *Klebsiella pneumoniae*

Rhodanese-catalysed restoration of activity of the inactive 2Fe species of the Fe protein

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The inactive 2Fe species of the Fe protein of the nitrogenase of *Klebsiella pneumoniae* was generated by treating oxidized Fe protein (Kp2) with MgATP and chelator. Incubation of the 2Fe species of Kp2 with the sulphurtransferase rhodanese in the presence of thiosulphate, ferric citrate and reduced lipoate reproducibly restored activity. The extent of restoration of activity depended on the molar ratio of 2Fe Kp2 to rhodanese and was time-dependent. Re-activation did not occur in the reaction mixture lacking rhodanese.

INTRODUCTION

During nitrogenase turnover, the Fe protein functions as a specific electron donor to the MoFe protein, the probable N_2 -binding site (see Thorneley & Lowe, 1984; Eady *et al.*, 1987). Purified Fe proteins from all diazotrophs have similar properties with an approximate M_r of 64000 and γ_2 subunit structure. The present consensus view is that these proteins contain a single [4Fe-4S] redox centre which spans the two identical subunits and traverses the +1 and +2 oxidation levels during the nitrogenase turnover cycle. This view of the Fe proteins as having a single iron-sulphur cluster has been questioned (Braaksma *et al.*, 1983), and these proteins have unusual e.p.r. properties where signals arising from both $S = \frac{1}{2}$ and $S = \frac{3}{2}$ spin states are associated with native $S_2O_4^{2-}$ -reduced Fe proteins (Hagen *et al.*, 1985; Watt & McDonald, 1985; Lindahl *et al.*, 1985). Attempts to learn more about the electron-transfer process in the Fe proteins and about the unusual properties of the redox centre have utilized the MgATP enhancement of Fe release from reduced and oxidized Fe proteins (Walker & Mortenson, 1974; Ljones & Burris, 1978; Anderson & Howard, 1984). Treatment of oxidized Av2 with the chelating agent $\alpha\alpha'$ -bipyridyl results in the formation of an inactive species containing a [2Fe-2S] centre (Anderson & Howard, 1984), which on reduction with $S_2O_4^{2-}$ forms an inactive 4Fe species (Howard *et al.*, 1985a).

Reconstitution studies of the Fe proteins lacking the full complement of iron could provide insight on factors involved in the formation of catalytically functional iron-sulphur centres. We therefore exploited the re-activation of inactive species of Kp2 deficient in Fe by the enzymic system based on the sulphurtransferase rhodanese. Rhodanese restores the activity in complex enzyme systems (Bonomi *et al.*, 1977; Pagani & Galante, 1983) and catalyses the reconstitution of both [2Fe-2S] and [4Fe-4S] clusters in apo-ferredoxins of both plant and bacterial origin (Pagani *et al.*, 1984; Bonomi *et al.*,

1985a). In the present paper we report that rhodanese catalyses the restoration of activity to the inactive 2Fe species of Kp2.

MATERIALS AND METHODS

Chemicals were reagent grade from Merck, Serva and BDH Chemicals.

Nitrogenase components of *Klebsiella pneumoniae* were purified and assayed as described by Eady (1980). Rhodanese was isolated from bovine liver by the method of Blumenthal & Henrikson (1971) and crystallized in the presence of thiosulphate. Thiosulphate reductase activity of rhodanese was measured as the initial rate of absorbance increase at 333 nm due to the formation of lipoic acid ($\epsilon_{333} = 150 \text{ M}^{-1} \cdot \text{cm}^{-1}$) (Volini & Westley, 1966). In this assay the activity of the enzyme ranged from 20 to 50 mol of lipoate oxidized/min per mol of enzyme in 0.1 M-Tris/HCl buffer, pH 8.0, containing 10–20 mM-thiosulphate and 20–40 μM -DL-dihydrolipoate at 20 °C.

DL-Dihydrolipoate prepared as described by Bonomi *et al.* (1985b) was determined with 5,5'-dithiobis-(2-nitrobenzoate) (Ellman, 1959). Acid-labile sulphide was determined by the method of Tijemchenko & Kimura (1979), and Fe content by the method described by Braaksma *et al.* (1983). Protein concentration was determined by using for rhodanese $\epsilon_{280} = 55750 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Cannella *et al.*, 1972), and for nitrogenase Fe proteins the micro biuret method.

Oxidized Kp2 (Kp2_{ox}) was prepared in a glove-box under N_2 containing less than 1 p.p.m. of O_2 . Kp2 (9.5 mg/ml) of specific activity 900–1120 nmol of acetylene reduced/min per mg of Kp2 was oxidized with Indigo Carmine bound to Dowex as described by Eady *et al.* (1987). Kp2_{ox} was separated from oxidation products by gel filtration on a Bio-Gel P-6DG column (0.6 cm $\times$ 20 cm) equilibrated with 50 mM-Tris/HCl buffer, pH 8.0, containing 100 mM-NaCl. The specific activity of the oxidized protein was 1337–1600 nmol of

Abbreviations used: Fe proteins of nitrogenase from different organisms are abbreviated as Kp2, *Klebsiella pneumoniae*, Av2, *Azotobacter vinelandii*, and Cp2, *Clostridium pasteurianum*.

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acetylene reduced/min per mg of Kp2 and it contained 4 mol of sulphide groups and 3.5–4 mol of Fe atoms per mol.

A 2Fe species of Kp2 was prepared from Kp2_{ox.} as described in the Results and discussion section.

RESULTS AND DISCUSSION

By following the strategy developed for the rhodanese-catalysed reconstitution of iron-sulphur centres in apo-ferredoxin polypeptides (Bonomi *et al.*, 1985a) we initiated studies on the re-activation of apo-Kp2 protein by rhodanese. Apo-Kp2 was prepared by following the procedure in which MgATP is used to facilitate chelation of Fe from the cluster by bathophenanthroline disulphonate (Ljones & Burris, 1978). Absorbance changes indicated that approximately 4 mol of Fe atoms/mol was removed from S₂O₄²⁻-reduced Kp2 when it was incubated anaerobically for 10–15 min with MgATP and chelator (results not presented, but see Fig. 1). Separation of protein from the incubation mixture by gel filtration under anaerobic conditions resulted in preparations of inactive apo-Kp2 containing less than 0.02 mol of sulphide groups/mol. Attempts to restore activity by incubation with rhodanese, thiosulphate, iron and reduced lipoate were sometimes, but not always, successful. However, even when inactive, rhodanese-treated Kp2 incorporated up to 4 mol of sulphide groups/mol. No significant incorporation of sulphide into rhodanese-treated Kp2 was observed when iron was omitted from the re-activation mixture, suggesting that formation of the iron-sulphur centre must take place for sulphide to be detected in the isolated protein.

Sulphide incorporation into the apo-Kp2 protein alone was not sufficient to restore activity, and other factors, which were not well characterized, were

Fig. 1. Release of Fe from Kp2 protein as measured by chelation with $\alpha\alpha'$ -bipyridyl in 50 mM-Tris/HCl buffer, pH 8.0, containing 100 mM-NaCl

The $\alpha\alpha'$ -bipyridyl concentration was 4 mM, MgATP 5 mM and Kp2_{ox.} 17 μ M. The data for Kp2_{red.} were obtained by adding to the Kp2_{ox.} before the addition of MgATP, S₂O₄²⁻ at 5 mM final concentration. Reactions were measured at 20 °C under an atmosphere of Ar. Curve 1, Kp2_{red.}; curve 2, Kp2_{red.} plus MgATP; curve 3, Kp2_{ox.}; curve 4, Kp2_{ox.} plus MgATP. Fe was quantified by using $\epsilon_{520} = 8.4 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ for the Fe- $\alpha\alpha'$ -bipyridyl complex (Walker & Mortenson, 1974).

Fig. 2. Absorbance spectra of the Kp2 species

The absorbance spectra were recorded anaerobically at room temperature in 1 cm-pathlength cuvettes in a Perkin-Elmer model lambda 5 spectrophotometer. Kp2_{ox.} (3.1 mg/ml; ----) and the 2Fe species of Kp2 (3.46 mg/ml; —) were prepared as described in the text.

important. Conformational constraints imposed by the presence of an appropriate even if inactive iron-sulphur centre might be required for restoration of activity.

The report by Anderson & Howard (1984) on the generation of an inactive 2Fe species of Av2 formed by treating oxidized Fe protein with MgATP and chelator prompted us to re-investigate rhodanese re-activation of Kp2 starting from a 2Fe species.

The iron-sulphur centre of the reduced Fe proteins Cp2 and Av2 is only susceptible to chelation in the presence of MgATP, which induces a conformational change in these proteins (Walker & Mortenson, 1974; Ljones & Burris, 1978; Braaksma *et al.*, 1983; Anderson & Howard, 1984). Comparison of curves 1 and 2 of Fig. 1 shows that Kp2 behaves similarly: in the absence of MgATP the rate of Fe chelation by $\alpha\alpha'$ -bipyridyl is very low, but in the presence of the nucleotide the reaction is essentially complete within 2 min. As reported for Av2 by Anderson & Howard (1984), the reaction pattern is markedly changed when oxidized Fe protein is used. Fig. 1 shows that both in the presence and in the absence of MgATP the reaction of Kp2_{ox.} with $\alpha\alpha'$ -bipyridyl is biphasic and that in the presence of the nucleotide the first phase is 40% complete within 5 min. Kp2, treated as above and recovered from the incubation mixture by gel filtration, contained 1.69 ± 0.22 mol of sulphide groups and 1.00 ± 0.4 mol of Fe atoms per mol. For different preparations the specific activity had decreased by approx. 95% from the starting value for Kp2_{ox.} of 1337–1600 nmol of acetylene reduced/min per mg of protein. The absorption spectrum of this material was different from that of Kp2_{ox.} (Fig. 2), and very similar to that of the 2Fe species of Av2 reported by Anderson & Howard (1984). Both 2Fe species have maxima in the visible region at 325, 416 and 460 nm, with A_{325}/A_{416} ratios of 1.45 and 1.42 and A_{416}/A_{460} ratios of 1.20 and 1.25 respectively. Generally these spectra are very similar to those of oxidized 2Fe-2S ferredoxins (see Orme-Johnson, 1973).

Fig. 3. Time course of the re-activation of the 2Fe species of Kp2

The 2Fe species of Kp2 (40–60 μ M) in 50 mM-Tris/HCl buffer, pH 8.0, containing 100 mM-NaCl was incubated with 8–10 mM-ferric ammonium citrate, 15–22 mM-thiosulphate and 40 mM-DL-dihydrolipoate at room temperature under Ar. Reconstitution was started by addition of rhodanese (1.3–6.3 μ M). At the times indicated samples of the reaction mixtures were taken and the nitrogenase activity was assayed as described by Eady (1980). The 2Fe Kp2/rhodanese molar ratios in the reconstitution experiments were the following: $\square$, 33:1; ▤ , 15:1; ▨ , 10:1; ▧ , 6:1. For comparison the time course of re-activation of the reaction mixture lacking rhodanese is also shown ($\blacksquare$).

Fig. 4. Thiosulphate reductase activity of rhodanese in the presence of holo-Kp2 and the 2Fe species of Kp2

Kp2 proteins and rhodanese in 50 mM-Tris/HCl buffer, pH 8.0, containing 80 mM-NaCl, 2 mM-thiosulphate and 10 mM-DL-dihydrolipoate were incubated anaerobically at room temperature. At the times indicated the thiosulphate reductase activity of rhodanese was assayed as described in the Materials and methods section in the presence of 10 mM-thiosulphate and 40 mM-DL-dihydrolipoate on samples of the incubation mixture. Protein concentrations in the incubation mixtures were the following: $\circ$, rhodanese, 0.42 mg/ml; $\diamond$, rhodanese, 0.8 mg/ml, plus the 2Fe species of Kp2, 13.0 mg/ml (2Fe Kp2/rhodanese

Re-activation of 2Fe species of Kp2

Attempts to restore activity to the 2Fe species of Kp2 by anaerobic incubation with Na_2S , mercaptoethanol and ferric citrate under conditions that reconstitute iron-sulphur centres in apo-ferredoxins (Hong & Rabinowitz, 1967) were unsuccessful. Incubation of the 2Fe species of Kp2 with rhodanese in the presence of thiosulphate, ferric citrate and reduced lipoate, conditions that result in the enzymic reconstitution of iron centres in apo-ferredoxins (Bonomi *et al.*, 1985a), reproducibly restored activity. The extent of restoration of activity was time-dependent, showing an increase to a maximum followed by a decrease (Fig. 3). It also depended on the molar ratio of 2Fe Kp2 to rhodanese. At a 2Fe Kp2/rhodanese ratio of 33:1 the maximum degree of restoration of activity was 29%, which increased to 66% (specific activity 670 nmol of acetylene reduced/min per mg of Kp2) at a ratio of 6:1. Re-activation was reproducible and did not occur in the reaction mixture lacking rhodanese.

Interaction of rhodanese with Kp2

Interaction between rhodanese and spinach ferredoxin resulted in an inhibition of the thiosulphate reductase

molar ratio 8:1); $\blacklozenge$, rhodanese, 0.42 mg/ml, plus the 2Fe species of Kp2, 15.0 mg/ml (2Fe Kp2/rhodanese molar ratio 18:1); $\square$, rhodanese, 0.42 mg/ml, plus holo-Kp2, 8.8 mg/ml (Kp2/rhodanese molar ratio 10:1).

activity of rhodanese. In contrast, the apo-ferredoxin species protected the rhodanese sulphurtransferase activity, which otherwise declined with time, probably as a result of the accumulation of lipoate, formed during the catalytic cycle (Pagani *et al.*, 1983).

Fig. 4 shows that the 2Fe species of Kp2, but not holo-Kp2, protected rhodanese from inactivation during turnover. Protection of rhodanese by interaction with (partial) apo-protein appears to represent a feature shared by both simple and complex iron-sulphur proteins; the overall pattern may represent a general mechanism of feedback regulation of the biosynthesis of iron-sulphur structures (Bonomi *et al.*, 1985a). If the lower activity of rhodanese in the presence of holo-Kp2 is due to inhibition, this may account for our observations that complete re-activation of Kp2 does not occur. The reason for the decrease in activity of Kp2 at longer incubation times (Fig. 3) is not clear. It is unlikely to be due to complex-formation between the two proteins, since Kp2 is present in a large excess over rhodanese; it may be due to the presence of oxidized lipoate formed during rhodanese catalysis. Further work will be required to determine the properties of the reconstituted Kp2. The data presented here constitute the first report of the enzymic restoration of activity to an inactive nitrogenase Fe protein 2Fe species. The variable results that we obtained with apo-Kp2 suggest that conformation constraints imposed by the presence of a 2Fe cluster in Kp2 facilitate the reconstitution of a functional 4Fe centre.

The *K. pneumoniae* *nifM*-gene product has a role in activating and stabilizing the *nifH*-gene product (the Kp2 polypeptide) (see Roberts *et al.*, 1978; Berman *et al.*, 1985; Howard *et al.*, 1985b). The biochemical nature of the activation process remains obscure, but the insertion of the iron-sulphur cluster in the right place is obviously necessary. The present study utilized mammalian rhodanese, but the results could indicate that the *nifM*-gene product may function as a *nif*-specific sulphurtransferase, conferring activity to the apo-Kp2 polypeptide.

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