Nitrogenase of *Klebsiella pneumoniae*: Electron-Paramagnetic-Resonance Studies on the Catalytic Mechanism

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The enzyme nitrogenase has been purified in different laboratories from a number of sources and studied extensively (Burris, 1971; Hardy *et al.*, 1971; Eady *et al.*, 1972). It consists of two proteins, the smaller containing iron and labile sulphur and the larger molybdenum, iron and labile sulphur. Turn-over of the enzyme involves transfer of reducing equivalents from $\text{Na}_2\text{S}_2\text{O}_4$ or other reducing substrates to the oxidizing substrate, which may be N_2 or some other triple-bonded molecules, or H^+ ions. The latter are always reduced to H_2 , although in the presence of other oxidizing substrates the rate of this reaction is inhibited to 20–25% of that in their absence. A bivalent cation, and ATP, which is hydrolysed to ADP, are always required.

The individual roles of the two proteins in the enzyme turnover process have not been definitely elucidated. Bui & Mortenson (1968), using co-chromatography of the proteins with $[^{14}\text{C}]\text{ATP}$ and K^{14}CN , found binding of ATP to the iron-containing protein, and of the reducible substrate cyanide to the molybdenum-and-iron-containing protein, of *Clostridium pasteurianum*. However, Biggins & Kelly (1970) found binding of ATP and cyanide to either protein from *Klebsiella pneumoniae*. Kelly & Lang (1970) found that binding of cyanide was enhanced when both proteins and ATP were present. This enhanced binding was inhibited by CO. Evans *et al.* (1971) studied the e.p.r. spectrum of the molybdenum-and-iron-containing protein of the nitrogenase from *Chloropseudomonas ethylicum*, though this may have been a mixture of at least two bacterial species (Gray *et al.*, 1972). In the $g = 1.94$ region they observed ATP-dependent changes on addition of KCN, which they correlated with those occurring on oxidation by $\text{K}_3\text{Fe}(\text{CN})_6$. The kinetics of these reactions were not reported.

The present communication reports kinetic e.p.r. studies on the molybdenum-and-iron-containing protein (Kp1) from *Klebsiella pneumoniae* (Eady *et al.*, 1972). The results show that reducing equivalents from $\text{Na}_2\text{S}_2\text{O}_4$ are transferred rapidly to protein Kp1 from the iron-containing protein, Kp2, in an ATP-dependent reaction. The process in which pro-

tein Kp1 is reoxidized by the N_2 molecule is probably rate-limiting in N_2 fixation.

Methods

The techniques for protein purification, oxidation of protein Kp1 with Lauth's Violet and manual mixing of samples for e.p.r. spectroscopy are described elsewhere (Eady *et al.*, 1972). E.p.r. spectra were recorded on a Varian E9 spectrometer, usually at 15°K and 20mW (Lowe *et al.*, 1972). g values were measured with diphenylpicrylhydrazine and Cr^{3+} -doped crystals of an ammonium-cobalt-EDTA complex as standards (Aasa *et al.*, 1966).

All protein and other component solutions were prepared under an atmosphere of N_2 gas in the hope that this, rather than H^+ ions, would be the major substrate in those experiments where actively fixing mixtures were prepared. All ATP solutions also contained MgCl_2 and an ATP-generating system of creatine phosphate and creatine kinase. The components of this mixture were always in the ratio used in the normal assay mixture (Eady *et al.*, 1972). In a typical experiment protein concentrations were 15–35 μM -Kp1 and 30–50 μM -Kp2. The rapid-freezing technique was that of Bray (1961). Anaerobic gel filtration to remove $\text{Na}_2\text{S}_2\text{O}_4$ was as described by Pick & Bray (1969). All experiments were carried out at room temperature (23–24°C).

Results and discussion

The e.p.r. spectrum of protein Kp1, as isolated in 1 mM- $\text{Na}_2\text{S}_2\text{O}_4$ by the method of Eady *et al.*, (1972), is similar to that of the corresponding protein from other sources (Burns *et al.*, 1970; Dalton *et al.*, 1971; Davis *et al.*, 1972). The main spectral component is of the rhombic type, with g values of 4.33, 3.68 and 2.015, the form being identical with Fig. 1 (top) of Davis *et al.* (1972). Additionally, resonances in the $g = 1.94$ region are sometimes detectable, though these may be due to impurities (Davis *et al.*, 1972).

Conversion of protein Kp1, as isolated (referred to below as state S), into other states of the molecule, numbered I–IV and distinguished by different e.p.r.

† Abbreviation: e.p.r., electron paramagnetic resonance.

Table 1. *States of the nitrogenase protein Kp1*

State S represents protein Kp1 as normally prepared in 1 mM- $\text{Na}_2\text{S}_2\text{O}_4$ by the method of Eady *et al.* (1972). The other states are obtained from it by the treatments indicated. E.p.r. signals correspond to the principal g values of the main components detected at 15°K.

State	Conditions	E.p.r. signals
S	Enzyme as normally prepared	$g = 2.015, 3.68, 4.33$
I	O_2 -inactivated	$g = 2.0, 4.3$
II	Oxidized with $\text{K}_3\text{Fe}(\text{CN})_6$	$g = 1.6\text{--}2.0, 4.3$
III	Oxidized with Lauth's Violet	Nil
IV	Reduced during steady state	Nil

Scheme 1. *Interconversions of the various states of protein Kp1 described in Table 1*

properties, may be achieved by the methods summarized in Table 1 and Scheme 1.

The O_2 -damaged form, I, was prepared by exposing state S to O_2 for 2½ h at 30°C. Protein Kp1 was irreversibly inactivated by this treatment and the product exhibited e.p.r. signals at $g = 4.3$ and 2.0. Similar spectra have been observed for the O_2 inactivated molybdenum-and-iron-containing proteins from other sources (Dalton *et al.*, 1971; Hardy *et al.*, 1971).

The oxidized form, II, was prepared by anaerobic titration of state S with $\text{K}_3\text{Fe}(\text{CN})_6$. A very complex spectrum appeared in the $g = 2.0\text{--}1.6$ region as the signal at $g = 3.68$ disappeared, but a line near $g = 4.3$ remained. Full activity was retained during this oxidation, and addition of $\text{Na}_2\text{S}_2\text{O}_4$ resulted in recovery of the e.p.r. spectrum of state S. We do not discuss states I and II further in the present communication since we have no evidence that they are involved in the catalytic mechanism.

Another oxidized form, III, was prepared by treatment of state S with the redox dye Lauth's Violet ($E_0 = +60\text{mV}$ at pH7). Its e.p.r. spectrum consisted solely of a small residual resonance near $g = 4.3$, probably due to an impurity, plus a weak free-radical signal. This state retained full enzymic activity and could be re-converted into state S, as discussed below. It should be noted particularly that state III was formed under oxidizing conditions and reverted to state S under reducing conditions. This distinguishes it unequivocally from state IV, which was also devoid of e.p.r. features. As described

below, state IV was formed under reducing conditions and reverted to state S under oxidizing conditions.

We next examined the e.p.r. spectra of the complete nitrogen-fixing system, concentrating our measurements on the $g = 3.68$ line, since this unusual e.p.r. feature is uniquely characteristic of protein Kp1 in state S. Under presumed steady-state conditions, we found that this resonance disappeared virtually completely (over 90%). The decrease in signal intensity did not take place if any of the components was omitted, even at concentrations of $\text{Na}_2\text{S}_2\text{O}_4$ up to 0.25M. Nor was there a decrease in signal intensity if ATP was replaced by ADP, a competitive inhibitor of nitrogen fixation by nitrogenase from *K. pneumoniae* (Parejko & Wilson, 1971). Further experiments were made on the complete system but with the supply of $\text{Na}_2\text{S}_2\text{O}_4$ (0.5 mM) limiting and working in the presence of an excess of ATP plus creatine phosphate (80 mM) and an excess of oxidizing substrates (N_2 0.7 mM; protons limited only by buffer, 25 mM). Here it was found that the $g = 3.68$ signal, which was diminished to 20% of its original intensity after 2 min, had reappeared after 10 min, presumably owing to exhaustion of the $\text{Na}_2\text{S}_2\text{O}_4$. When the $\text{Na}_2\text{S}_2\text{O}_4$ concentration was raised to 1.5 mM, the signal was not detected at 2 min or 10 min but had reappeared after 60 min. It was concluded that under steady-state conditions protein Kp1 is converted almost quantitatively into state IV and that this represents a highly reduced form of the molecule.

The above results imply a turnover time much longer than the optimum value of 185 ms/2 electrons per molecule of protein Kp1 at 30°C as calculated from the results of Eady *et al.* (1972). However, this is consistent with the known effects on the reaction rate of sub-optimum concentrations of protein Kp2 and $\text{Na}_2\text{S}_2\text{O}_4$ (Eady *et al.*, 1972) together with temperature (Hardy *et al.*, 1971) and the high ionic strength necessary to maintain the MgCl_2/ATP ratio (Shah *et al.*, 1972).

Unfortunately, we were unable to reproduce the production of state IV in rapid-freezing experiments,

presumably owing to the difficulties of using this method anaerobically and because state IV autooxidized readily to state S. This precluded fast kinetic studies on the conversion of state S into state IV. However, we were able to study in some detail an analogous reaction, the conversion of the oxidized state III into state S.

$\text{Na}_2\text{S}_2\text{O}_4$ alone reduced state III slowly to state S with quantitative recovery of the e.p.r. spectrum. Addition of protein Kp2 or ATP separately did not enhance the rate, but when added together they caused very fast reduction to state S. In rapid-freezing experiments, when protein Kp2 and ATP in 10mM- $\text{Na}_2\text{S}_2\text{O}_4$ solution were mixed with state III, reduction to state S, as monitored by appearance of the $g = 3.68$ signal, occurred with a half-time of approx. 10ms. When reduced protein Kp2 (from which $\text{Na}_2\text{S}_2\text{O}_4$ had been removed by anaerobic gel filtration) was used as the sole reductant in the presence of ATP, very rapid reduction of protein Kp1 in state III to state S was also observed. This showed that protein Kp2 could donate electrons rapidly to protein Kp1.

Since reduction of state S to state IV, like that of state III to state S, was dependent on the presence of ATP and protein Kp2 as well as on a supply of reducing equivalents, it seems reasonable to assume that these two reactions are entirely analogous to one another, with regard to both the rate of reaction and the role of protein Kp2. If this is accepted it is then possible to formulate the overall turnover reaction as one of ATP-mediated transfer of reducing equivalents from $\text{Na}_2\text{S}_2\text{O}_4$ via protein Kp2 to protein Kp1. Re-oxidation of protein Kp1 would then be accomplished by the oxidizing substrate, which was mainly N_2 in our experiments. If reduction of state S to state IV is anything like as fast as that of state III to state S, then the former cannot be rate-limiting in enzyme turnover. Hence the process in which protein Kp1 is reoxidized must be rate-limiting, and this is indeed consistent with the observed greater-than-90% reduction of state S to state IV in the steady state.

The above interpretation will require confirmation by anaerobic rapid-freezing experiments. The mechanism of ATP involvement also requires clarification, as does the nature of the e.p.r. chromophore of state S of protein Kp1, the precise nature of the other states and the numbers of electrons involved in the various reactions.

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