Biochem. J. (1973) **133**, 405–408
Printed in Great Britain

Nitrogenase of *Klebsiella pneumoniae*: Evidence for an Adenosine Triphosphate-Induced Association of the Iron–Sulphur Protein

By ROGER N. F. THORNELEY and ROBERT R. EADY
Agricultural Research Council Unit of Nitrogen Fixation, University of Sussex, Brighton BN1 9QJ, Sussex, U.K.

(*Received* 12 *March* 1973)

The effect of various nucleotides on the Fe-containing component of nitrogenase of *Klebsiella pneumoniae* was investigated by ultracentrifugation and thiol-group reactivity towards 5,5′-dithiobis-(2-nitrobenzoate). In the absence of $Na_2S_2O_4$, ATP and ADP produced changes in sedimentation behaviour and thiol-group reactivity consistent with association of the protein.

Nitrogenase specifically requires ATP for activity (Moustafa & Mortenson, 1967; Hardy *et al.*, 1968; Eady *et al.*, 1972); although several hypotheses for the mode of action of ATP have been proposed (see Hardy & Burns, 1968), little experimental evidence exists to support them. Of the two proteins that comprise nitrogenase, an Fe-containing protein and an Fe+Mo-containing protein (see Burris, 1971), ATP has been shown to interact with the former. [^{14}C]ATP has been used to demonstrate binding of ATP to Cp2 protein* (Moustafa & Mortenson, 1969) and Ac2 protein (Yates, 1972), although Biggins & Kelly (1970) demonstrated binding to both Kp1 and Kp2 proteins and considered it to be non-specific. E.p.r. (electron-paramagnetic-resonance) studies strongly supported the possibility of an interaction between the Fe-containing protein and ATP (Zumft *et al.*, 1972). Kinetic e.p.r. studies have demonstrated a requirement of ATP for the transfer of electrons from Kp2 protein to Kp1 protein (Smith *et al.*, 1972), from Cp2 protein to Cp1 protein (Zumft *et al.*, 1972; Orme-Johnson *et al.*, 1972) and from Av2 protein to Av1 protein (Orme-Johnson *et al.*, 1972). The present communication presents data that demonstrate the specific binding of ATP to Kp2 protein, thereby inducing the formation of higher-ordered complexes from Kp2 subunits, a process that is reversed on the addition of $Na_2S_2O_4$.

Methods

Nitrogenase proteins were prepared and assayed as described by Eady *et al.* (1972). Because $Na_2S_2O_4$ and dithiothreitol react with 5,5′-dithiobis-(2-nitrobenzoate) it was necessary to remove them from Kp2 protein by anaerobic chromatography on Sephadex G-25. The protein prepared in this way had an e.p.r. spectrum that showed a resonance at $g = 1.94$, which is characteristic of iron–sulphur proteins in the reduced state (Tsibris & Woody, 1970), and is absent from Kp2 protein in its oxidized form (B. E. Smith, personal communication); it had a specific activity of 1100nmol of ethylene produced/min per mg of protein when assayed under the conditions described by Eady *et al.* (1972). The extreme O_2-sensitivity of $Na_2S_2O_4$-free Kp2 protein (Eady *et al.*, 1972) necessitated the use of rigorous anaerobic techniques in order to retain enzymic activity. Solutions were degassed on a vacuum line to which argon (Puragon; Air Products Ltd., Hythe, Southampton, U.K.), which had been additionally purified by passage through an Oxysorb cartridge (Messer Griesheim Ltd., London SE27 0RH, U.K.), could be admitted. The O_2 content of the argon determined by the method of Sweetser (1967) was less than 0.5p.p.m. All-glass syringes and stainless-steel needles were used for solution transfers. An American Instrument Co. stopped-flow apparatus incorporating a log-photometer was used to follow the reactions. The output signal (absorbance) was displayed simultaneously on a Tektronix 549 storage oscilloscope and a Bryans 27000 chart recorder, enabling both the rapid reaction and the subsequent slow reaction to be monitored. The observation-chamber compartment was flushed with high-purity N_2 during the experiments and the observation chamber (1cm path length) was soaked overnight with 0.1M-$Na_2S_2O_4$ to remove dissolved O_2 from the Teflon and Kel-F components that came into contact with the reactants. Sedimentation coefficients were determined by using a Martin Christ 70000 preparative ultracentrifuge fitted with an analytical attachment, an An-D rotor at 20°C and schlieren optics being used. No loss of activity occurred during centrifugation of Kp2 protein in the

* The nitrogenase components of various organisms are denoted by a capital letter indicating the species and the number 1 or 2 indicating which of the protein components is referred to. The number 1 indicates the Fe+Mo-containing protein and number 2 the Fe-containing protein. Kp is *Klebsiella pneumoniae*, Cp is *Clostridium pasteurianum*, Av is *Azotobacter vinelandii* and Ac is *Azotobacter chroococcum*.

presence or in the absence of ATP. However, the addition of ATP to Kp2 protein produced a 50% decrease in specific activity before the loading of the centrifuge cell. This may be due to some inactivation by O_2 occurring during the transfer procedures. Yates (1972) has demonstrated that ATP induced hypersensitivity of Ac2 protein to inactivation by O_2.

Nucleotides and 5,5′-dithiobis-(2-nitrobenzoate) were purchased from Sigma (London) Chemical Co., Kingston-on-Thames KT2 7BH, Surrey, U.K., and were used without further purification. ATP was not detectable (less than 1%) in the ADP and AMP by t.l.c. in isobutyric acid–aq. NH_3 (sp.gr. 0.88)–water (66:1:33, by vol.) on cellulose F.

Results and discussion

Effect of ATP on thiol-group reactivity. We have used ultracentrifugation and the thiol-group reagent 5,5′-dithiobis-(2-nitrobenzoate) to investigate changes in conformation and quaternary structure of Kp2 protein exposed to ATP and other nucleotides. The Kp2 protein molecule (mol.wt. 66800) contains 4 iron and 4 acid-labile sulphide atoms with 18 cysteine residues and consists of two identical subunits (Eady *et al.*, 1972). The progress curve for the reaction of 5,5′-dithiobis-(2-nitrobenzoate) with Kp2 protein, monitored by the release of the thiophenylate ion, is shown in Fig. 1. Kinetically the reaction is complex, consisting of a spectrum of at least four exponentials, and detailed analysis of these rates is not discussed here. ATP at 0.5mM markedly accelerated the rate of reaction, the equivalent of 14 thiol groups/molecule reacting within 500s (see Fig. 1). Stopped-flow oscillographs indicated that even the most reactive thiol groups were subject to this acceleration. ADP (0.5mM), an inhibitor of nitrogenase action, produced a small but significant increase in the reactivity of the thiol groups of Kp2 protein to 5,5′-dithiobis-(2-nitrobenzoate) (Fig. 1). AMP, CTP, GTP, UTP or ITP (0.5mM) had no effect. The rate of reaction of O_2-damaged Kp2 protein with 5,5′-dithiobis-(2-nitrobenzoate) varied with the time-interval between exposure to air and reaction with the thiol-group reagent. Kp2 protein exposed to air for 30s reacted at the same rate as did native protein; after 300s exposure to air the reaction profile was intermediate between those of native Kp2 protein and Kp2 protein plus ATP; after 500s the titre was 9 thiol-group equivalents/molecule (see Fig. 1).

Effect of ATP on the sedimentation behaviour of Kp2 protein. ATP, in the presence of $Na_2S_2O_4$, did not alter the sedimentation behaviour of Kp2 protein; values of about 4.6S were obtained, consistent

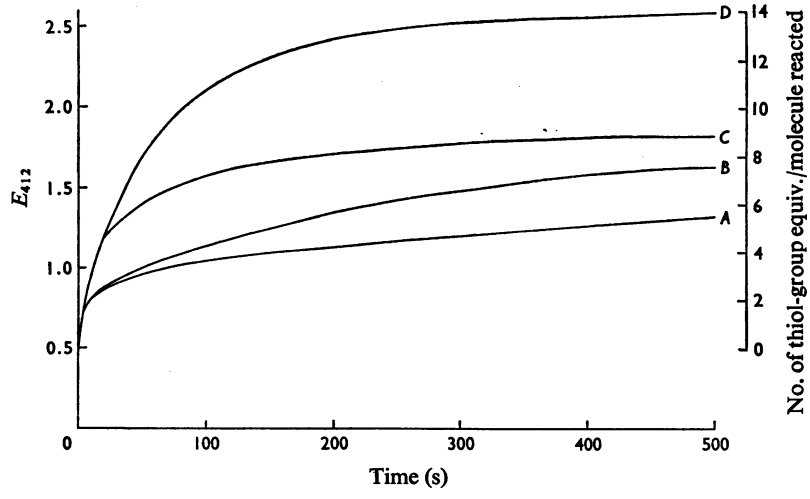

Fig. 1. *Effect of ATP on the reaction of 5,5′-dithiobis-(2-nitrobenzoate) with Kp2 protein*

The figure represents a stopped-flow spectrophotometric chart record at 412nm of the reaction of 5,5′-dithiobis-(2-nitrobenzoate) (final concn. 1.25mM) with Kp2 protein (final concn. 0.70mg/ml, 10.5μM assuming mol.wt. 66800). One syringe contained Kp2 protein and the other 5,5′-dithiobis-(2-nitrobenzoate) alone or 5,5′-dithiobis-(2-nitrobenzoate) and ATP or ADP at a concentration of 1mM. Both syringes contained Tris–HCl buffer, pH7.4 (25mM), and $MgCl_2$ (10mM). The reactions were carried out at 25°C under strictly anaerobic conditions. Reactions followed were: curve *A*, Kp2 protein with 5,5′-dithiobis-(2-nitrobenzoate); curve *B*, Kp2 protein with 5,5′-dithiobis-(2-nitrobenzoate)+ADP; curve *C*, Kp2 protein, after exposure to air for 300s, with 5,5′-dithiobis-(2-nitrobenzoate); curve *D*, Kp2 protein with 5,5′-dithiobis-(2-nitrobenzoate)+ATP.

with the $s^0_{20,w}$ value of 4.7S obtained previously for the protein alone (Eady *et al.*, 1972). In the absence of $Na_2S_2O_4$, ATP evoked a marked change in the sedimentation pattern of Kp2 protein, producing, in addition to the normal 4.6S component, a well-resolved faster-sedimenting component of sedimentation coefficient 6.6S (Fig. 2). Assuming values of 0.69ml/g for the apparent partial specific volume and $D^0_{20,w}$ of 5.55×10^{-7}cm^2/s (Eady *et al.*, 1972) to apply, these sedimentation coefficients correspond to approximate molecular weights of 64000 and 91000. Although under our conditions these peaks have sedimentation coefficients attributable to the dimer and trimer of the Kp2 protein subunit, this correlation could well be fortuitous, since in a system undergoing rapid equilibration there is generally no simple correlation between sedimentation coefficient and molecular weight and between the number of peaks observed in the ultracentrifuge and the number of species present in solution.

$Na_2S_2O_4$ (final concn. 1mM) suppressed the formation of the faster-sedimenting component when added to ATP and $Na_2S_2O_4$-free Kp2 protein, and a single peak of sedimentation coefficient 4.5S was observed (Fig. 2). ADP (0.5mM) caused Kp2 protein in the absence of $Na_2S_2O_4$ to sediment as an assymmetric peak of sedimentation coefficient 7.2S (Fig. 2). AMP, CTP, GTP or ITP (0.5mM) did not change the sedimentation behaviour of the Kp2 protein.

Because ATP produces hypersensitivity to O_2, resulting in inactivation of preparations of highly purified Ac2 protein (Yates, 1972), the effect of damage by O_2 on the sedimentation behaviour of Kp2 protein was investigated. O_2-inactivated Kp2 protein sedimented as a single peak of sedimentation coefficient 4.5S and the addition of ATP produced an additional peak of sedimentation coefficient 8.6S; these sedimentation coefficients, with the reservations noted above, correspond to approximate molecular weights of 64000 and 120000.

In our experiments the nucleotides were present as their Mg^{2+} complexes, and it is likely that it is these Mg^{2+} complexes of the nucleotides that produce the effects noted above.

Conclusion

The increase in thiol titre from 6 to 14 thiol-group equivalents/molecule in the presence of ATP could be attributed to an ATP-induced dissociation of the two protein subunits of Kp2 protein, before formation of higher-order complexes of the subunits, as indicated by the ultracentrifuge experiments, occurs. The 14 thiol groups/molecule that eventually react with 5,5′-dithiobis-(2-nitrobenzoate) is a greater number than that observed with the protein when denatured with 8M-urea, where about 10 thiol groups/molecule react (Eady *et al.*, 1972). This increase is possibly due to damage to the iron–sulphur chromophore in the presence of 5,5′-dithiobis-(2-nitrobenzoate) and ATP, resulting in the release of iron with concomitant exposure of additional thiol groups or S^{2-}, both of which react with 5,5′-dithiobis-(2-nitrobenzoate).

The increase in titre from 6 to 8 thiol groups/molecule after 500s in the presence of ADP indicates that ADP also interacts with the Kp2 protein. This result, and the less-well-resolved asymmetric peak seen in ultracentrifuge experiments, suggests that ADP produces a similar effect to that of ATP, possibly inducing the same association of protein subunits but with different rate and equilibrium constants.

These findings indicate that, in the absence of

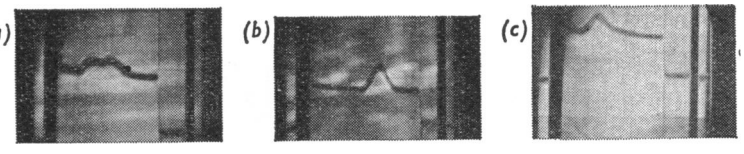

Fig. 2. *Ultracentrifuge schlieren patterns of Kp2 protein showing the effects of ATP, ADP and ATP plus $Na_2S_2O_4$ on sedimentation behaviour*

The pictures were taken at 70, 75 and 65min after reaching a speed of 60000rev./min in a Martin Christ 70000 preparative ultracentrifuge fitted with an analytical attachment. Sedimentation at 20°C was from right to left under a gas phase of N_2. (*a*) Kp2 protein (3.1mg of protein/ml) in 25mM-Tris–HCl buffer, pH7.4, containing 0.5mM-ATP and 10mM-$MgCl_2$. (*b*) Kp2 protein under similar conditions with 1mM-$Na_2S_2O_4$ added. The specific activity measured after the addition of ATP was 480nmol of ethylene produced/min per mg of protein. (*c*) Kp2 protein (4.6mg of protein/ml) in 25mM-Tris–HCl buffer, pH7.4, containing 0.5mM-ADP and 10mM-$MgCl_2$.

$Na_2S_2O_4$, ATP and to a smaller extent ADP induce the formation of a number of higher-ordered polymers (*n*-mers) of the Kp2 protein subunit, but are insufficient to formulate them with certainty. Because their formation is induced by one co-substrate (ATP) and also by an inhibitory product (ADP) of nitrogenase and is suppressed by the presence of a second, albeit unnatural, co-substrate ($Na_2S_2O_4$), *n*-mers of Kp2 protein may well be important in the catalytic cycle of nitrogenase function. A knowledge of their relative concentrations and rates of interconversion is a prerequisite for the detailed analysis of both steady state and pre-steady-state kinetic data.

We thank Professor J. R. Postgate for useful discussion and comments on the manuscript, Dr. B. E. Smith for helpful discussion and Mrs. E. Morris and Mr. B. Vyse for technical assistance.

Biggins, D. R. & Kelly, M. (1970) *Biochim. Biophys. Acta* **205**, 288–299

Burris, R. H. (1971) in *The Chemistry and Biochemistry of Nitrogen Fixation* (Postgate, J. R., ed.), chapter 4, Plenum Press, London

Eady, R. R., Smith, B. E., Cook, K. A. & Postgate, J. R. (1972) *Biochem. J.* **128**, 655–675

Hardy, R. W. F. & Burns, R. C. (1968) *Annu. Rev. Biochem.* **37**, 331–358

Hardy, R. W. F., Holsten, R. D., Jackson, E. K. & Burns, R. C. (1968) *Plant Physiol.* **43**, 1185–1207

Moustafa, E. & Mortenson, L. E. (1967) *Nature (London)* **216**, 1241–1243

Moustafa, E. & Mortenson, L. E. (1969) *Biochim. Biophys. Acta* **172**, 106–115

Orme-Johnson, W. H., Hamilton, W. D., Jones, T. L., Tso, M.-Y. W., Burris, R. H., Shah, V. K. & Brill, W. J. (1972) *Proc. Nat. Acad. Sci. U.S.* **69**, 3142–3145

Smith, B. E., Lowe, D. J. & Bray, R. C. (1972) *Biochem. J.* **130**, 641–643

Sweetser, P. B. (1967) *Anal. Chem.* **39**, 979–982

Tsibris, J. C. M. & Woody, R. W. (1970) *Coordin. Chem. Rev.* **5**, 417–458

Yates, M. G. (1972) *Eur. J. Biochem.* **29**, 386–392

Zumft, W. G., Cretney, W. C., Huang, T. C., Mortenson, L. E. & Palmer, G. (1972) *Biochem. Biophys. Res. Commun.* **48**, 1525–1532