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Nitrogenase of *Klebsiella pneumoniae*

INTERACTION OF THE COMPONENT PROTEINS STUDIED BY ULTRACENTRIFUGATION

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Sedimentation-velocity analyses of mixtures of the component proteins of nitrogenase of *Klebsiella pneumoniae* at a 1:1 molar ratio, showed a single peak of sedimentation coefficient (12.4S) considerably greater than that obtained for the larger (Fe+Mo-containing) protein centrifuged alone (10.4S). When the ratio exceeded 1:1 (the smaller Fe-containing protein in excess) an additional peak corresponding in sedimentation coefficient (about 4.5S) to free Fe-containing protein appeared. When proteins, which had been inactivated by exposure to air were used, no interaction occurred. $Na_2S_2O_4$ at 2mM both reversed and prevented interaction between the two proteins; sedimentation coefficients corresponded to those of the proteins when centrifuged alone. These results demonstrate the formation of a complex between the nitrogenase proteins, and, together with data of activity titration curves, are consistent with the formulation of the nitrogenase complex of *K. pneumoniae* as (Fe-containing protein)–(Fe+Mo-containing protein).

Nitrogenase from a number of organisms has been fractionated into two protein components, an Fe-containing protein and an Fe+Mo-containing protein (see Burris, 1971). The Fe-containing protein of *Klebsiella pneumoniae* (Kp2; nomenclature of Eady *et al.*, 1972) has a molecular weight of 66800 in solution and is made up of two subunits of molecular weights 34600. The Fe+Mo-containing protein (Kp1; nomenclature of Eady *et al.*, 1972) has two different subunits of molecular weights 50000 and 60000 present in equal numbers and a solution molecular weight of 218000 (Eady *et al.*, 1972). Both proteins are required for nitrogenase activity but neither protein has been demonstrated to catalyse partial reactions involving either ATP or reducible substrates. Kinetic e.p.r. (electron-paramagnetic-resonance) studies have demonstrated a role for the Fe-containing protein in the ATP-dependent transfer of electrons to the Fe+Mo-containing protein (Smith *et al.*, 1972; Orme-Johnson *et al.*, 1972; Zumft *et al.*, 1972).

Evidence for the existence of a more than transient complex (with a life-time longer than the 180ms turnover time per electron pair; Smith *et al.*, 1972) between the two proteins during nitrogenase action is derived from steady-state kinetic studies, which show a disproportionate decline in activity at high dilutions (Silverstein & Bulen, 1970; Yates, 1970) and sigmoidal plots of activity when the Fe+Mo-containing protein is titrated with Fe-containing protein (Bergersen & Turner, 1973). In the present paper sedimentation-velocity experiments are described that demonstrate the formation of a single complex between Kp1 protein and Kp2 protein over a wide range of ratios where Kp2 protein is in excess, and constitute the first unambiguous physical demonstration of a complex-formation between the component proteins of nitrogenase.

Materials and Methods

Nitrogenase proteins were purified and assayed as described by Eady *et al.* (1972). $Na_2S_2O_4$-free proteins were prepared by anaerobic chromatography on Sephadex G-25; the extreme O_2-sensitivity of the proteins under these conditions (Eady *et al.*, 1972) necessitated the use of rigorous anaerobic techniques to retain enzymic activity. An all-glass vacuum line was used to de-gas solutions, which were then gassed with argon (Puragon; Air Products Ltd., Hythe, Southampton, Hants., U.K.), which was further purified by scrubbing through a gas-train of photo-reduced Methyl Viologen (Sweetser, 1967) to remove O_2. The solutions were accessible through Suba-Seal (Griffin and George, Wembley, Middx., U.K.) closures which, if used unpierced under vacuum and under positive gas pressure when pierced, gave satisfactory results. The O_2 content of the gas was less than 0.5p.p.m. as measured by the method of Sweetser (1967). The dissolved O_2 concentration in de-gassed buffers, measured by using electrolytically reduced Methyl Viologen and an anaerobic stopped-flow spectrophotomoter, was less than 1μM (R. N. F. Thorneley, personal communication). De-gassed Tris–HCl buffer, pH7.4, containing 10mM-$MgCl_2$ was delivered directly to the head of a column (1cm×20cm) of Sephadex G-25 by gas pressure from the de-gassing vessel. Solutions were handled

in all-glass syringes with no gas headspace. By using this apparatus $Na_2S_2O_4$-free nitrogenase components at concentrations about 10μM could be prepared with full activity and stored for up to 6h at room temperature.

The $Na_2S_2O_4$-free proteins used in this work had specific activities of 1130nmol of ethylene formed/min per mg of protein for Kp1 protein and 890nmol of ethylene formed/min per mg of protein for Kp2 protein when assayed as described by Eady *et al.* (1972).

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.

Results and Discussion

The extreme O_2-sensitivity of Kp1 protein and Kp2 protein (Eady *et al.*, 1972) necessitates the inclusion of 1mM-$Na_2S_2O_4$ in buffers used in the preparation of these proteins to avoid inactivation by O_2. Sedimentation-velocity analyses of mixtures of Kp1 protein and Kp2 protein in 25mM-Tris–HCl buffer, pH7.4, containing 10mM-$MgCl_2$ and 2mM-$Na_2S_2O_4$ gave values of sedimentation coefficients characteristic of Kp1 and Kp2 proteins when sedimenting alone (see Table 1). Fig. 1 shows schlieren patterns obtained in typical experiments performed at a variety of molar ratios of the two proteins. The leading boundary corresponded in sedimentation coefficient to Kp1 protein, and the tailing boundary to Kp2 protein, indicating that no interaction between the proteins occurs under these conditions. The s values were in good agreement with the $s^0_{20,w}$ values of 11S for Kp1 protein and 4.8S for Kp2 protein obtained previously (Eady *et al.*, 1972). There was no indication of dissociation of Kp1 protein from a tetramer to a dimer at concentrations below 5mg of protein/ml as is observed with Cp1 protein (nomenclature of Eady *et al.*, 1972) (Huang *et al.*, 1973).

If $Na_2S_2O_4$ was absent from such mixtures of Kp1 and Kp2 proteins, then, under otherwise identical conditions, pronounced changes in sedimentation behaviour were observed. Sedimentation-velocity analysis of a mixture of Kp1 protein and Kp2 protein at a molar ratio of 1:1 gave a single slightly asymmetrical boundary (see Fig. 1), with a sedimentation coefficient of 12.4S, significantly greater than the value of 10.5S obtained for Kp1 protein alone at the same concentration. At higher ratios of Kp2 protein to Kp1 protein (with Kp2 protein in excess) the reaction boundary became symmetrical and an additional slower-sedimenting symmetrical boundary with a sedimentation coefficient of about 4.5S appeared. The latter boundary corresponds in sedimentation coefficient with free Kp2 protein. A theoretical treatment of interacting macromolecules undergoing mass transport as in sedimentation-velocity experiments (Gilbert & Jenkins, 1959) indicates that a faster-sedimenting boundary should be regarded only as a qualitative representation of complex-formation. In this instance, it is a reaction boundary at which the equilibrium Kp1 protein + Kp2 protein $\rightleftharpoons$ Kp1–Kp2 complex is continually readjusted during differential transport of the complex, Kp1 and Kp2 proteins. In addition, the sedimentation coefficient of this boundary should be greater than that of either component protein of nitrogenase, but less than that of the complex were it to exist alone. Fig. 1 shows schlieren patterns obtained in typical experiments at a variety of molar ratios of the two proteins. The sedimentation coefficient of the reaction boundary did not change significantly over a range of Kp2 concentrations from equimolar with Kp1 protein, to conditions where Kp2 protein was in a sevenfold excess of Kp1 protein (Table 1). Since there was no

Table 1. *Sedimentation coefficients of boundaries in mixtures of Kp1 protein and Kp2 protein*

Sedimentation coefficients were determined at 20°C in 25mM-Tris–HCl buffer, pH7.4, containing 10mM-$MgCl_2$ and where indicated, 2mM-$Na_2S_2O_4$, under argon. The ratio of Kp2 protein to Kp1 protein was varied and molar ratios were calculated by using molecular weights of 66800 for Kp2 protein and 218000 for Kp1 protein (Eady *et al.*, 1972).

Kp1 concn. (mg of protein/ml)	Kp2 concn. (mg of protein/ml)	Molar ratio Kp1/Kp2	Sedimentation coefficient of leading boundary		Sedimentation coefficient of tailing boundary	
				$+Na_2S_2O_4$		$+Na_2S_2O_4$
3.5	1.08	1:1	12.4	—	No peak	—
2.8	1.05	1:1.2	12.8	10.4	No peak	4.4
2.7	2.58	1:3.1	12.3	10.6	4.6	4.5
2.7	6.03	1:7.3	12.75	10.7	4.4	4.1
—	3.1		4.6	4.4		
2.8	—		10.5	10.8		

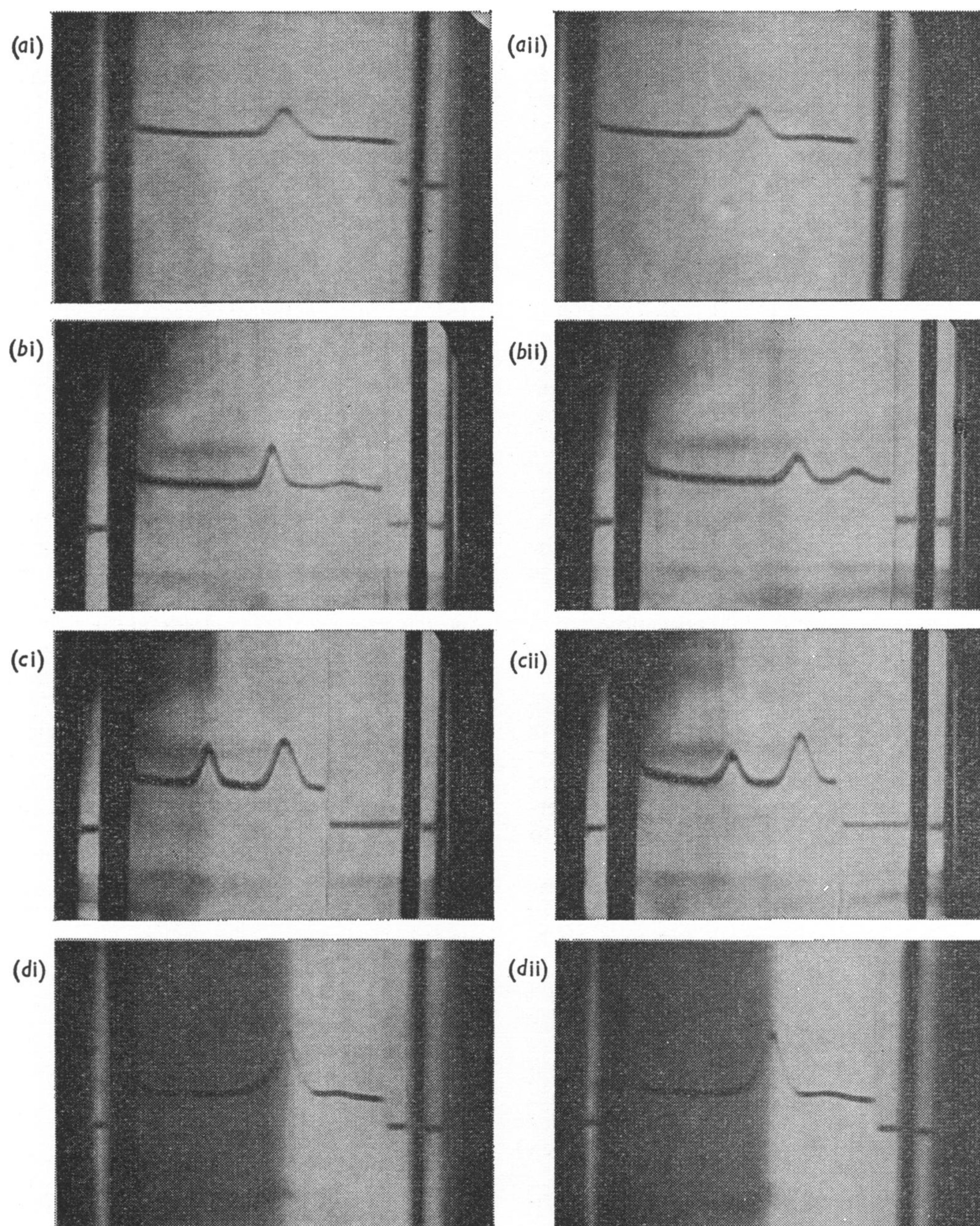

Fig. 1. *Ultracentrifuge schlieren patterns of mixtures containing Kp1 protein and Kp2 protein, showing the effect of the addition of $Na_2S_2O_4$*

The pictures were taken at 30min after reaching 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 argon. The proteins were in 25mM-Tris–HCl buffer, pH7.4, containing 10mM-$MgCl_2$ and, where indicated, 2mM-$Na_2S_2O_4$. The protein concentrations are those of Table 1 at corresponding molecular ratios, except for (*d*) where the protein concentrations were: Kp1 protein, 9.7mg/ml; Kp2 protein, 1.3mg/ml. The molar ratios of Kp2 protein to Kp1 protein are (*a*) 1:1, (*b*) 1:3.6, (*c*) 1:7.2, (*d*) 0.45:1. The schlieren diaphragm angle was 50°, except for (*d*) 55°. Mixtures in (*a* i), (*b* i), (*c* i) and (*d* i) contain no $Na_2S_2O_4$ and those in (*a* ii), (*b* ii), (*c* ii) and (*d* ii) contain $Na_2S_2O_4$.

boundary corresponding to free Kp2 protein when Kp1 and Kp2 protein were equimolar, this behaviour is consistent with the formulation of the complex as 1:1 (Kp1 protein to Kp2 protein). Evidently under these conditions, complex-formation between Kp1 protein and Kp2 protein is favoured over a wide range of protein ratios, and even with a large excess of Kp2 protein a single 1:1 complex predominates.

Under conditions where Kp1 protein was in excess of Kp2 protein, the sedimentation pattern, although indicating interaction between the two proteins, was more difficult to interpret. Two poorly resolved boundaries were seen, a rather broad tailing boundary and an asymmetric leading boundary with a sedimentation coefficient of 11.6S. On the addition of 2mM-$Na_2S_2O_4$ the leading boundary became symmetrical, the area under it decreased by 20% (see Fig. 1*d*) and the sedimentation coefficient became 10.4S. It is possible that the asymmetry of the leading edge of the leading boundary observed in the absence of $Na_2S_2O_4$ is due to some complex of the type (Kp2 protein)–(Kp1 protein)$_n$ being formed. The existence of such a complex was postulated (Eady *et al.*, 1972) to account for the inhibition observed in activity titration curves when Kp1 protein is in excess of Kp2 protein.

O_2-damaged Kp1 protein and Kp2 protein centrifuged anaerobically under conditions where native proteins interact did not form a complex. A rather broad poorly resolved double boundary was seen, with the sedimentation coefficient of the faster-sedimenting peak (9.4S) probably corresponding to free Kp1 protein.

Activity titration curves for nitrogenase components of *K. pneumoniae* show an optimum molar ratio of 1:1 for reducible substrates other than KCN (Eady *et al.*, 1972, 1973). The assignment of a stoicheiometry to a nitrogenase complex from such data alone is questionable, since, although the molar ratios of the two proteins are known at each point in the titration curve, the amount of each protein actually complexed is not. However, the existence of a sharp optimum in activity at an integral molar ratio of 1:1 or 1:2 would imply tight binding between the two proteins.

The nitrogenase complex of *Clostridium pasteurianum* has been assigned the type (Mo+Fe-containing protein)–(Fe-containing protein)$_2$, because activity titration curves have an optimum at a protein Cp2/Cp1 molar ratio of 2:1 (Burris, 1969; Vandecasteele & Burris, 1970; Tso *et al.*, 1972). However, Mortenson *et al.* (1973) obtained curves with a maximum activity at a protein Cp2/Cp1 molar ratio of 1:1, but proposed a 2:1 composition for the complex because of the sigmoidal behaviour of activity when the Cp2 concentration was increased at a fixed Cp1 concentration. Bergersen & Turner (1973) also postulated the formation of a 2:1 complex for soya-bean root nodule bacteroid nitrogenase. They obtained sigmoidal curves for plots of $V'_{C_2H_2}$ and V'_{NH_3} against Fe–protein concentration and derived interaction coefficients of about 2 for the Fe-protein binding sites. The activity titration curves for nitrogenase components of *K. pneumoniae* (Eady *et al.*, 1972) are unusual in that they show a very sharp inhibition of nitrogenase activity if the concentration of Kp1 protein is in excess of Kp2 protein. Similar titration curves for *C. pasteurianum* (Ljones & Burris, 1972) and *Azotobacter chroococcum* (M. G. Yates, personal communication) show much wider optima before inhibition occurs.

It could be that the complex formed by the interaction of Kp1 protein and Kp2 protein which occurs at the protein concentrations used in the ultracentrifuge experiments is fortuitous, and not relevant to that formed at normal assay concentrations, especially since its formation is only observed in the absence of $Na_2S_2O_4$, a normal component of the assay system. However, the stoicheiometry of the complex determined from the ultracentrifuge data is consistent with that indicated by titration studies for *K. pneumoniae*, i.e. a 1:1 complex, and activity measurements made at high protein concentrations show no significant differences from assays made with dilute proteins (Smith *et al.*, 1973). The effect of $Na_2S_2O_4$ in preventing complex-formation may be related to its effect on Kp2 protein, since it has been shown to suppress the ATP-induced changes of the state of association of Kp2 protein under these conditions (Thorneley & Eady, 1973).

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