RESEARCH COMMUNICATION

Nitrogenase of *Klebsiella pneumoniae*: kinetics of formation of the transition-state complex and evidence for an altered conformation of MoFe protein lacking a FeMoco centre

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We have investigated the kinetics of inactivation of Mo-nitrogenase isolated from *Klebsiella pneumoniae* when it forms an inhibited putative transition-state complex on incubation with ADP and AlF_4^- . In the presence of excess Kp2 (Fe protein of the Mo-nitrogenase of *K. pneumoniae*), the kinetics were found to depend on the Mo content of Kp1 (the MoFe protein of Mo-nitrogenase of *K. pneumoniae*). The residual nitrogenase activity versus time of incubation using Kp1 preparations containing integral, i.e. one or two Mo atoms per molecule of Kp1, were essentially monophasic, but significantly different rates of inactivation were observed. In contrast, the progress curves for preparations of Kp1 with non-integral Mo content were biphasic, suggesting the presence of two discrete catalytically active species

of Kp1. The best fit to the observed data was obtained with a two-exponential expression, the amplitude of which was consistent with the Mo content, provided that the fast phase of the reaction was assigned to a Kp1 species containing one, and the slow phase to a species containing two Mo atoms per $\alpha_2\beta_2$ tetramer. This analysis provides the first evidence for the existence of a catalytically active Kp1 species containing a single Mo atom. These data also indicate that MoFe protein which does not have all FeMoco binding sites occupied has an altered conformation compared with a fully loaded protein, and that the Fe protein reacts with these conformations at different rates to form the stable, but inhibited transition-state complex.

INTRODUCTION

The formation of a transient electron-transfer complex between the Fe protein and the MoFe protein of nitrogenases is an integral part of the catalytic cycle of the enzyme (see [1,2] for reviews). In this complex, the transfer of one electron from the Fe protein to the MoFe protein is accompanied by the hydrolysis of two molecules of ATP, but at the present time the mechanism of coupling of ATP hydrolysis to electron transfer and substrate reduction is not clear. Recently conditions have been established resulting in the formation of a stable complex of nitrogenase components of *Azotobacter vinelandii* (Av1, Av2). This complex has the stoichiometry $\text{Av1}:(\text{Av2})_2$ and is formed when nitrogenase turnover occurs in the presence of MgATP and AlF_4^- [3] or when both proteins are incubated in the presence of MgADP and AlF_4^- [4]. The complex, which is inactive in both substrate reduction and ATP hydrolysis, has been proposed to be a stable analogue of a putative transition-state complex or a transient intermediate in nucleotide hydrolysis. Subsequently, we demonstrated that nitrogenase components of *Klebsiella pneumoniae* (Kp1, Kp2) formed a similar complex under pseudo-turnover conditions in the presence of MgADP and AlF_4^- [5]. The solution structure of the Kp complex at ~ 1.5 nm (15 Å) resolution has been determined from synchrotron X-ray scattering using a model-independent shape restoration method. This analysis showed that the Fe protein bound to the $\alpha\beta$ dimeric half-molecule of the MoFe protein, such that the 2-fold symmetric surface surrounding the $[4\text{Fe}4\text{S}]$ centre of the Fe protein is the contact surface to the pseudo-symmetric interface of $\alpha\beta$ subunits of the MoFe protein. This docking model had been proposed by Rees and co-workers, based on the individual X-ray crystal

structures of Av1 and Av2, biochemical and genetic data [6–8]. This model has subsequently been confirmed from a crystallographic study of the transition-state complex of *Azotobacter* nitrogenase, where the conformation of the Fe protein is seen to be altered as a consequence of complex formation [9]. In the present work we sought to characterize the kinetics of the rate of inactivation of the Mo-nitrogenase from *K. pneumoniae* during the formation of a putative transition-state complex on incubation with ADP and AlF_4^- . During the course of these studies we observed biphasic kinetics for the inactivation reaction when the preparations of Kp1 used contained non-integral amounts of Mo. A kinetic analysis of these data are consistent with Kp1 assuming an altered conformation of the $\alpha_2\beta_2$ tetramer when the cofactor-binding site located in a single α subunit is not occupied. This change appears to alter the rate of complex formation with Fe protein and the $\alpha\beta$ dimeric half-molecule which contains FeMoco.

MATERIALS AND METHODS

$\text{AlF}_3 \cdot \text{H}_2\text{O}$ (Aldrich), MgCl_2 (Fisons), sodium dithionite (BDH Merck), KF and K-ADP (potassium ADP) (Sigma) were used as supplied. Kp1 and Kp2 were prepared as described in [10] and [11] respectively. Metal analyses of preparations of Kp1 were carried out using inductively coupled plasma analyses by Southern Sciences Ltd., Brighton, BN1 9PY, U.K. on samples wet-ashed as described in [11]. In order to generate AlF_4^- , $\text{AlF}_3 \cdot \text{H}_2\text{O}$ and KF were mixed and dissolved in water, as described below, before addition to the buffered incubation mixture as described previously [4].

Abbreviations used: Kp1, MoFe protein of Mo-nitrogenase of *K. pneumoniae*; Kp2, Fe protein of Mo-nitrogenase of *K. pneumoniae*; Av1, MoFe protein of Mo-nitrogenase of *A. vinelandii*; Av2, Fe protein of Mo-nitrogenase of *A. vinelandii*; P, the Fe_8S_8 iron–sulphur cluster present in reduced MoFe proteins.

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Formation of the transition-state complex

The Kp1–AlF₄–Kp2 complex was prepared in 50 mM Hepes buffer (pH 7.4) by incubating Kp1 and Kp2 with 7.5 mM MgCl₂/5.0 mM KADP/20 mM Na₂S₂O₄, containing ~ 5 mM AlF₄[–], in a final volume of 2 ml. The amounts of Kp1 used were: 4.8 μM Kp1 (containing 1.9 Mo/molecule), 18.8 μM Kp1 (containing 1.1 Mo/molecule) or 15.6 μM Kp1 (containing 1.41 Mo/molecule); the amount of Kp2 used was 7.5 × [Kp1]. A ~ 70 mM stock solution of AlF₄[–] was prepared by mixing appropriate amounts of AlF₃ and KF in water so that their final concentrations were 0.07 M and 0.7 M respectively. After extensive stirring, the resulting suspension was filtered and stored at –20 °C. In a typical kinetic experiment, appropriate volumes of the AlF₃/KF, Hepes, MgCl₂ and KADP solutions were mixed together and argon gas was bubbled through the solution for about 30 min. Dithionite (200 μl) was added followed by Kp1 and Kp2, and the reaction mixture was incubated at room temperature under argon. At regular time intervals, 20 or 25 μl samples were removed from the incubation mixture and assayed for acetylene reduction activity.

The acetylene reduction assay was conducted according to published procedures [10]. The specific activity of the enzyme is expressed as nmol of acetylene reduced/min per mg of Kp1.

Data analysis

The molar concentration of the catalytically active sites of Kp1, designated as [Kp1]* which are left uncomplexed at any time *t*, was determined by using the following equation:

$$\text{Molar concn.} = \text{SA}_t/\text{SA}_0 \times 228000 \times \text{number of Mo atoms} \quad (1)$$

where SA_{*t*} = specific activity at time *t*, SA₀ = specific activity at time *t* = 0 and 228000 = molecular mass of Kp1. If it is assumed that a Kp1 species A, containing one cofactor, and a species B, containing two cofactors, react at different rates to give the catalytically inactive species C (where C = C_A + C_B), then:

If the initial concentration of A is A₀ and that of B is B₀, then eqns. (4) and (5) apply:

$$A = A_0 e^{-k_1 t} \quad (4)$$

$$B = B_0 e^{-k_2 t} \quad (5)$$

According to mass balance, C = A₀ – A + B₀ – B and C_∞ = A₀ + B₀, thus:

$$\log(C_\infty - C) = \log(A_0 e^{-k_1 t} + B_0 e^{-k_2 t}) \quad (6)$$

where (C_∞ – C) = [Kp1]*.

Eqn. (6) was used to develop theoretical traces by allowing A₀, B₀, *k*₁ and *k*₂ to change freely until the best fit through the experimental points was obtained.

RESULTS AND DISCUSSION

The X-ray crystal structures of MoFe proteins revealed that the metal content of preparations with a full complement of redox centres should be 2 Mo and 30 Fe atoms per tetramer [8]. In practice, preparations often contain a lower metal content than this ideal value. Quantification of the Mossbauer spectra of protein preparations of Kp1 [12], MoFe protein of Mo-nitro-

genase of *Clostridium pasteurianum* (Cp1) and Av1 [13], but with different metal contents, was consistent with FeMoco and P (the Fe₈S₈ iron–sulphur cluster present in reduced MoFe proteins) centres being present together in equivalent amounts in preparations isolated from wild-type strains, with typical Mo/Fe ratios of approx. 1:15 (see [13]). This ‘all-or-none’ hypothesis does not appear to apply in the case of mutated proteins, since the analytical and activity data for Kp1 mutated in Cys ligands to the P clusters are consistent with the existence of a species of Kp1 containing an excess of FeMoco over P centres. However, the excess amount of FeMoco in the dithionite-reduced state does not exhibit the characteristic *S* = 3/2 EPR spectrum of MoFe proteins and is catalytically inactive [10]. One thing which remains unclear is whether preparations of MoFe proteins isolated from wild-type organisms and which contain non-integral amounts of Mo are a mixture of holoprotein and apoprotein, or if a species of a MoFe protein containing a single FeMoco and P centre is also present. In this paper we present kinetic evidence for the existence of such a species with a single occupancy of the FeMoco and P cluster binding sites.

When preparations of Kp1 with a non-integral Mo content, in this case Kp1 containing 1.4 Mo atoms per α₂β₂, hereafter designated as Kp(1.4), were used for formation of the inhibited transition-state complex by reacting it with Kp2, MgADP and AlF₄[–], a non-linear, biphasic trace was observed (Figure 1). Two possibilities can be considered. First, that such preparations of Kp1 are a mixture of a fully-loaded holoprotein (i.e. Kp1 containing two FeMoco centres per α₂β₂ tetramer) and apoprotein (Kp1 containing no FeMoco). In this case, the biphasic rate of reaction may arise from a difference in reactivity of the two FeMoco centres of the fully loaded protein or from one site reacting to bring about conformational changes in the other site, which then reacts at a different rate. If this were the case, then irrespective of the reason for the difference between the rates of reaction of the two centres, the two reaction phases would be of equal amplitude.

The second possibility is that such preparations of Kp1 contain a mixture of a fully loaded holoprotein and a half-loaded protein (i.e. Kp1 in which one αβ subunit pair contains FeMoco, while in the other αβ pair the FeMoco-binding site is empty). The two phases would then arise from the different reaction rates of the two type of molecules. In such case, the amplitude of the two phases will depend upon the relative amounts of the two type of molecules.

Unfortunately, the two possibilities cannot be differentiated unequivocally from the data presented in Figure 1. However, when the inhibition kinetics of Kp1 preparations containing 1.1 and 1.9 Mo atoms/molecule of Kp1 were measured, a clearer situation emerged. The data of Figure 2 show that the amplitude of the fast phase decreased, while that of the slow phase increased when the Mo content of Kp1 increased from 1.1 to 1.9. The dependence of the amplitude of the two phases on Mo content of Kp1 clearly indicates that the first possibility discussed above does not apply. We consider that these data are best explained if such preparations of Kp1 contain two catalytically active, but discrete, species of Kp1, a suggestion consistent with the kinetic analysis discussed below.

The data of Figure 2 shows that Kp1 containing 1.9 and 1.1 Mo atoms/α₂β₂ [hereafter designated as Kp1(1.9) and Kp1(1.1) respectively] give rise to essentially monophasic traces. However, the rate of Kp1(1.1) is significantly faster than that of Kp1(1.9). In contrast, the kinetics for Kp1(1.4) is biphasic (Figure 1), and both the fast and slow phases of the reaction have significant amplitudes. This leads us to assign the fast phase of the reaction to those Kp1 molecules which contain only one

Figure 1 Time course for the formation of the inhibited transition-state complex with Kp1(1.4)

The incubation mixture (under an atmosphere of argon), contained Kp1 (15.6 μM , 1.4 Mo/molecule), 109 μM Kp2, 7.5 μM MgCl_2 , 5 μM KADP, $\sim 5 \mu\text{M}$ AlF_4 and 20 mM $\text{Na}_2\text{S}_2\text{O}_4$. The data points are the measured nitrogenase activity of samples removed at various times after initiation of the reaction to form the transition-state complex, as described in the Materials and methods section. The continuous line is a simulated fit to the activity data using eqn. (6).

Figure 2 Effect of the Mo content of Kp1 on the rate of formation of the inhibited transition-state complex

The incubation mixture was as described in the legend to Figure 1, except that the concentrations of Kp1 and Kp2 were (a) 4.8 μM and 36 μM respectively, and (b) 18.8 μM and 141 μM respectively. The data points are the measured nitrogenase activity for (a) Kp1(1.9) and (b) Kp1(1.1) determined at various times after initiation of the reaction to form the transition-state complex, as described in the Materials and methods section. The continuous lines are the simulated fits to the activity data using eqn. (6).

FeMoco, and the slow phase to Kp1 molecules which have both FeMoco sites fully occupied.

This assignment is further supported by our theoretical analysis. The continuous lines drawn through the experimental points in Figures 1 and 2, are the simulated fits of eqn. (6). These traces yield the values of the amplitudes, A_0 and B_0 , of the two phases which were used to calculate the percentages of the two type of molecules in each Kp1 preparation. As can be seen from Table 1, the calculated Mo contents compare very well with the experimentally determined values provided that the slow phase of the reaction is assigned to Kp1 molecules, which contain two FeMoco per $\alpha_2\beta_2$ tetramer, and the fast phase to molecules which

contain only one FeMoco per $\alpha_2\beta_2$ tetramer. In contrast, if the slow phase of the reaction is assigned to Kp1 molecules which contain one FeMoco per molecule and *vice versa*, the calculated Mo contents differ significantly from the observed values.

It is important to emphasize that the analysis we have used assumes that the two catalytically active sites of the fully loaded Kp1 molecules are inactivated at the same rate. Although this is consistent with the data for the Kp1 preparations shown in Figures 1 and 2, small differences in the rate of reaction, which may exist between these species, might not have been detected with the methods used in this study. The theoretical fits of the data to eqn. (6) also yield the values of the rate constants

Table 1 Measured Mo contents of three Kp1 preparations determined by metal analyses are compared with the Mo content computed by theoretical fits of the observed inhibition traces generated by eqn. (6)

A_0 and B_0 are the active site concentrations while k_1 and k_2 are the rate constants of formation of the inactive complex of Kp1 containing one or two occupied FeMoco sites respectively. The calculated Mo contents of Kp1 were obtained from the values of A_0 and B_0 indicated.

Kp1 species	% of Kp1 containing one FeMoco per $\alpha_2\beta_2$ tetramer	A_0 (M)	k_1 (s^{-1})	B_0 (M)	k_2 (s^{-1})	No. of Mo atoms/ $\alpha_2\beta_2$ tetramer of Kp1		
						Measured	Calculated by assigning fast phase to Kp1 with 1 FeMoco	Calculated by assigning fast phase to Kp1 with 2 FeMoco
Kp1(1.1)	93.6	4.21×10^{-6}	0.21	5.76×10^{-7}	1.3×10^{-2}	1.1	1.06	1.79
Kp1(1.4)	51.9	2.14×10^{-6}	0.56	3.96×10^{-6}	8.0×10^{-3}	1.4	1.48	1.21
Kp1(1.9)	17.7	8.00×10^{-7}	0.81	7.46×10^{-6}	3.5×10^{-3}	1.9	1.82	1.05

k_1 and k_2 . Values of these rate constants, together with those of A_0 and B_0 and the initial concentrations of Kp1 containing one or two occupied FeMoco centres respectively, are given in Table 1. Since the simulation of the data are sensitive to the values of A_0 and B_0 , reliable values of the percentage composition of Kp1 preparations and their theoretical Mo contents are obtained.

The data presented above provide the first direct experimental evidence for the existence of a discrete species of Kp1 which contains a single unoccupied FeMoco binding site and which has an altered reactivity. Our explanation for the modified reactivity is that when a single α subunit has the binding site for FeMoco unfilled, the reactivity of the FeMoco centre in the functional $\alpha\beta$ half molecule is changed and, since the separation of these two centres is 70 Å, this must be mediated by a conformational change across the $\alpha\beta$ dimer contact surfaces. We propose that the Fe protein senses the altered conformation of the α subunit of the MoFe protein when the cofactor-binding site, buried some 10 Å from the protein surface, is not occupied. This alteration in the rate of reaction of MoFe protein with different Mo content, to form the putative transition-state complex under pseudo-turnover conditions, may be important during turnover conditions. It also shows that preparations with a non-integral Mo content do not necessarily arise from a mixture of apoMoFe protein and fully functional MoFe protein, but may be due to the presence of Kp1 containing a single but fully catalytically active FeMoco centre per $\alpha_2\beta_2$ tetramer. Further work is required to determine whether the pre-steady-state electron-transfer reactions, which

occur in nitrogenase turnover, are altered as a consequence of this species of the MoFe protein which has an altered conformation.

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