Nitrogenase of *Klebsiella pneumoniae*

A STOPPED-FLOW STUDY OF MAGNESIUM-ADENOSINE TRIPHOSPHATE-INDUCED ELECTRON TRANSFER BETWEEN THE COMPONENT PROTEINS

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Stopped-flow kinetic data have been obtained for a rapid electron-transfer reaction between the component proteins of nitrogenase from *Klebsiella pneumoniae*, which was induced by MgATP. Up to three equivalents of the Fe-containing protein were rapidly oxidized by one equivalent of the Fe–Mo-containing protein in a unimolecular reaction, $k_2 = 2 \times 10^2 \text{ s}^{-1}$. Evidence for a tight complex between the component proteins, $K_{D(\text{complex})} < 0.5 \mu\text{M}$, which was formed with a rate $k_1 > 1 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$, has been obtained. MgATP bound to either the Fe-containing protein or to the two-protein complex with a rate $k_3 > 2.5 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ and with $K_{D(\text{MgATP})} = 0.4 \text{ mM}$, before the electron-transfer reaction.

Pre-steady-state kinetic data for electron-transfer reactions involving nitrogenase from *Klebsiella pneumoniae* (Smith *et al.*, 1972, 1973) and *Clostridium pasteurianum* (Mortenson *et al.*, 1973; Zumft *et al.*, 1974) have been obtained by the ‘rapid-freeze’ e.p.r.* technique (Bray *et al.*, 1973). Technical difficulties and the necessity for large amounts of purified proteins presumably prevented any detailed kinetic analysis by the above workers of the observed electron-transfer processes. However, it was concluded that for *Klebsiella pneumoniae* nitrogenase, a MgATP-induced electron transfer occurs from Kp2 to Kp1 protein with a half-life of 5–15 ms, which is much faster than the turnover time of the enzyme (Smith *et al.*, 1973). This electron transfer was monitored by the synchronous partial bleaching of the characteristic e.p.r. signals from both Kp1 and Kp2 proteins. When the system became exhausted of $\text{Na}_2\text{S}_2\text{O}_4$, the Kp1 protein signal returned but the Kp2 protein signal became completely bleached. This was interpreted as the production of a ‘super-reduced’ form of Kp1 protein during turnover. Mössbauer studies (Smith & Lang, 1974) further supported this theory. Orme-Johnson *et al.* (1972), with similar e.p.r. data for Cp proteins, came to the same conclu-

sions as Smith *et al.* (1973). Walker & Mortenson (1973) have recently accepted the ‘super-reduced’ theory, Mortenson *et al.* (1973) having previously proposed an oxidation of Cp1 protein in the steady state.

Eady *et al.* (1972) reported changes in the u.v. and visible spectra of the Kp proteins on oxidation by O_2 and the dye Lauth’s Violet. Changes in the spectra of Cp proteins have been used to determine both the redox potentials of the proteins (Walker & Mortenson, 1973) and the direction of electron flow under steady-state and substrate-limiting conditions, i.e. on exhaustion of $\text{Na}_2\text{S}_2\text{O}_4$ (Ljones, 1973). No spectrophotometric stopped-flow kinetic data have previously been reported for electron-transfer reactions in the ‘full fixing’ nitrogenase system. Kinetic data are presented in this paper of a rapid electron-transfer reaction, induced by MgATP, involving Kp1 and Kp2 proteins. Comparisons with the e.p.r. ‘rapid freeze’ data of Smith *et al.* (1972) are made and further evidence has been obtained for the ‘tight’ complex between Kp1 and Kp2 proteins proposed by Eady (1973).

Materials and Methods

The nitrogenase component proteins were purified as described by Eady *et al.* (1972) with an additional final DEAE-cellulose chromatography involving a linear elution gradient, 1 litre of 30–90 mM-MgCl₂ in 25 mM-Tris-HCl buffer, pH 8.7 (Smith *et al.*, 1974). Kp1 protein had a specific activity of 2000 nmol of C_2H_4 produced/min per mg of protein and 22 ± 2 Fe atoms and 1.7 ± 0.1 Mo atoms/218000 mol.wt. Kp2

* Abbreviations: e.p.r., electron paramagnetic resonance. The nitrogenase components of the various organisms are denoted by a capital letter indicating the genus, a lower-case letter indicating the species and the number 1 or 2 denotes which of the protein components is referred to. The number 1 indicates the Mo–Fe-containing protein and the number 2 the Fe-containing protein. Kp, *Klebsiella pneumoniae*; Cp, *Clostridium pasteurianum*; Av, *Azotobacter vinelandii*; Ac, *Azotobacter chroococcum*.

protein had a specific activity of 1100nmol of C_2H_4 produced/min per mg of protein and 3.5 ± 0.2 Fe atoms/67000 mol.wt. Stock protein solutions were prepared by dialysis for 5h against 50mM-Tris-HCl buffer, pH7.4, 10mM-MgCl₂, 10mM-Na₂S₂O₄, saturated with N₂ at 1×10^2 kPa (1atm). ATP (disodium salt), creatine kinase and creatine phosphate [Sigma (London) Chemical Co., Kingston-on-Thames KT2 7BH, Surrey, U.K.] were used without further purification. Stopped-flow experiments were done at 23°C, monitored at 425nm, in a 1cm-light-path cell with solutions, after mixing, containing, except where stated, 50mM-Tris-HCl buffer, pH7.4, 5mM-ATP, 10mM-MgCl₂, 10mM-Na₂S₂O₄, 12mM-creatine phosphate, 0.15mg of creatine kinase/ml. Anaerobic dilutions of stock solutions were done with gas-tight syringes into N₂-flushed vessels fitted with rubber serum-cap closures. Modifications to a commercial stopped-flow apparatus (American Instrument Co., Silver Spring, Maryland, U.S.A.) necessary to improve the temperature control and anaerobicity have been described (Thorneley, 1974). Absorbance-time traces displayed on a storage oscilloscope were photographed on 35mm film. The negative was projected back on to the oscilloscope screen with careful matching of the gratitudes. The projected image was then matched with an exponential curve generated on the oscilloscope. The generated curve, whose amplitude, vertical and horizontal position and time-constant (τ) could be independently varied, allowed several rapid determinations of τ to be made by using slightly different amplitudes and interpretations of 'noise'. Successive determinations on a single trace typically gave values of τ within $\pm 5\%$. Another advantage of this procedure, particularly important in this study, was that deviations from a

single exponential-decay process, due to either multiple exponential processes, i.e. concurrent or consequential electron transfers, or second-order kinetic processes, would have been readily detected.

Results

The time-constants (τ) and amplitudes for a rapid change in absorbance at 425nm for the 'full fixing' nitrogenase system as a function of protein concentrations, order of addition and dithionite concentration, are given in Table 1. A typical oscilloscope trace is shown in Fig. 1(a). In all cases a single exponential process was observed with a time-constant of 8.5 ± 1.0 ms. The amplitude and τ were both independent of the order of addition. When either Kp1 protein, Kp2 protein or ATP were omitted, no reaction was observed. When the Na₂S₂O₄ concentration was increased to 35mM, no significant change in τ or amplitude occurred. Increasing the concentration of Kp2 protein from 5 to 83 μ M with a fixed Kp1 protein concentration of 5 μ M caused the amplitude to increase (Fig. 2), but the τ remained constant. Increasing the Kp1 concentration from 5 to 23 μ M, with a constant Kp2 protein concentration of 5 μ M, had no effect on either the amplitude or time-constant. After remaining constant for several minutes, the absorbance returned to the original value observed immediately after mixing (Fig. 1b). These aged solutions were still strongly reducing and calculations indicated that the system would have run out of ATP and creatine phosphate after these time-intervals.

The dependence of τ and the amplitude on the MgATP concentration is given in Table 2. The concentration of MgATP was calculated by assuming

Table 1. Stopped-flow data for the MgATP-induced electron transfer from Kp2 to Kp1 protein in N₂-saturated buffer, pH7.4, 23°C

Unless otherwise stated both syringes also contained 50mM-Tris-HCl, pH7.4, 10mM-MgCl₂ and 10mM-Na₂S₂O₄. Final ATP concentration was 5mM in all cases. Values are means $\pm$ s.d. for the numbers of run indicated.

Kp2 (μ M)	Kp1 (μ M)	τ (ms)	ΔE_{425}	No. of runs	Comments	
					Syringe A	Syringe B
5.4	5.4	8.5 ± 0.5	0.010 ± 0.001	3	Kp1+Kp2	ATP
5.4	5.4	9.2 ± 0.2	9.012 ± 0.001	3	Kp1	Kp2+ATP
5.4	5.4	9.0 ± 0.5	0.011 ± 0.001	4	Kp1+ATP	Kp2
5.0	5.0	7.8 ± 0.3	0.011 ± 0.001	6	Kp1+Kp2	ATP
10.4	5.0	9.5 ± 1.0	0.021 ± 0.002	12	Kp1+Kp2	ATP
16.0	5.0	9.0 ± 1.0	0.028 ± 0.002	6	Kp1+Kp2	ATP
20.7	5.0	9.0 ± 1.0	0.029 ± 0.002	6	Kp1+Kp2	ATP
41.5	5.0	8.7 ± 1.0	0.032 ± 0.002	8	Kp1+Kp2	ATP
83.1	5.0	7.5	0.032	1	Kp1+Kp2	ATP
5.0	23.5	8.0	0.010 ± 0.001	2	Kp1+Kp2	ATP
19.8	20.7	8.0	0.035 ± 0.002	2	Kp1+Kp2	ATP
20.7	5.0	10.5 ± 1.0	0.025 ± 0.002	4	Kp1+Kp2	ATP+60mM-Na ₂ S ₂ O ₄

Fig. 1. Stopped-flow oscillograms obtained on mixing Kp1 and Kp2 proteins with MgATP in the presence of Na₂S₂O₄ at 23°C, pH 7.4, λ 425 nm

Final concentrations after mixing were: Kp1 protein, 5 μM; Kp2 protein, 5 μM; MgCl₂, 10 mM; Na₂S₂O₄, 10 mM; Tris-HCl, 50 mM. Oscillogram (a) for [MgATP] 4.1 mM and (b) for [MgATP] 0.063 mM. The horizontal trace in (b) was obtained by manual triggering after about 3 min when the ATP, but not the Na₂S₂O₄, was exhausted and the absorbance had returned to the value observed immediately after mixing.

Fig. 2. Effect on the amplitude at 425 nm of increasing the ratio Kp2/Kp1 proteins

The Kp1 protein concentration was kept constant at 5 μM and the Kp2 protein concentration increased from 5 to 83 μM. Data are taken from Table 1. Over this range of concentrations τ remained constant at 8.5 ± 1.0 ms.

Fig. 3. Dependence of τ on the reciprocal of MgATP concentration

Data are taken from Table 2.

Table 2. Dependence of τ on MgATP concentration

[Kp1] was 5 μM with [Kp2] at 5 μM. Proteins were mixed in the stopped-flow apparatus with MgATP, creatine phosphate and creatine kinase at 23°C. Both syringes also contained 10 mM-MgCl₂, 10 mM-Na₂S₂O₄, 50 mM-Tris-HCl, pH 7.4. The reaction was monitored in a 1 cm-light-path cell at 425 nm. Values are means ± s.d.

[MgATP] (mM)	τ (ms)	ΔE ₄₂₅
4.1	8.3 ± 0.5	0.010 ± 0.001
1.4	7.8 ± 0.5	0.011 ± 0.001
0.66	8.0 ± 0.5	0.013 ± 0.001
0.32	9.0 ± 1.0	0.012 ± 0.001
0.19	16.0 ± 1.0	0.012 ± 0.001
0.13	21.0 ± 1.0	0.011 ± 0.001
0.063	39.0 ± 1.0	0.011 ± 0.001
0.063*	40.0 ± 1.0	0.029 ± 0.002

* [Kp2] = 20 μM

K₁ = 0.11 mM, K₂ = 16 mM and K₃ = 40 mM for the dissociation constants of MgATP, Mg₂ATP and Mg-creatine phosphate respectively, as discussed by Thorneley & Willison (1974). A single exponential process was observed with a constant amplitude for equimolar Kp1 and Kp2 proteins (5 μM). When, at the lowest MgATP concentration, the Kp1/Kp2 protein ratio was increased from 1:1 to 1:4, the amplitude increased by a factor of 3 in exactly the manner observed at the highest MgATP concentration (Table 1, Fig. 2). A plot of τ (1/k_{obs}) versus 1/[MgATP] (Fig. 3) was linear up to 0.66 mM-MgATP. At higher concentrations of MgATP, τ remained constant at 8 ± 0.5 ms. In all cases after the system exhausted the ATP supply, the absorbance returned to the value observed immediately after mixing (Fig. 1b).

Discussion

The increase in absorbance occurring with τ = 8.5 ± 1 ms is almost certainly due to the MgATP-induced oxidation of Kp2 protein. Increases in

absorbance in the region 380–700nm have been reported for the oxidation of both Kp1 and Kp2 proteins (Eady *et al.*, 1972). However, e.p.r. (Smith *et al.*, 1973) with Mössbauer (Smith & Lang, 1974) studies have shown that in the steady state, Kp2 protein becomes oxidized and Kp1 protein further reduced. Rapid-freeze e.p.r. data (Smith *et al.*, 1973) indicated a synchronous oxidation of Kp2 protein and reduction of Kp1 protein with $\tau = 7.3$ ms when Kp2 plus Kp1 proteins were mixed with MgATP. This value is very close to $\tau = 8.5 \pm 1$ ms obtained in the present study. However, Smith *et al.* (1973) obtained a value of $\tau = 22$ ms when Kp1 protein plus MgATP were mixed with Kp2 protein. Changing the order of addition of Kp1 protein, Kp2 protein or MgATP did not effect either τ or amplitude. Zumft *et al.* (1974), in rapid-freeze e.p.r. studies involving Cp1 protein, Cp2 protein and MgATP, also observed times that were independent of the order of addition. Further evidence for the increase in absorbance being due to oxidation of Kp2 protein is that on increasing the concentration of Kp2 protein at constant Kp1 protein concentration, the amplitude increased (Fig. 2), but on increasing the Kp1 protein concentration at constant Kp2 protein concentration, the amplitude remained constant (Table 1). If the total amplitude is associated with the oxidation of Kp2 protein, i.e. ignoring changes in absorbance of Kp1 protein, then for Kp1/Kp2 ratios of 1:1, 1:2 and 1:3 the $\Delta\epsilon_{425}/\text{mol}$ of Kp2 protein = $2000 \text{ M}^{-1} \cdot \text{cm}^{-1}$. None of the amplitudes has been corrected for the effect of the 1.5 ms dead-time of the stopped-flow apparatus. For a reaction with $\tau = 8.5$ ms, 85% of the total amplitude would be observed. Eady *et al.*, (1972) reported $\Delta\epsilon_{425} = 4000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for the O_2 inactivation of Kp2 protein in the absence of MgATP.

Under second-order conditions for a bimolecular reaction i.e. $[\text{Kp1}] = [\text{Kp2}]$, a single first-order exponential process was observed. The time-constant (τ) was independent of both Kp1 and Kp2 protein concentrations. For equivalent concentrations of Kp1 and Kp2 proteins, a scheme involving rapid complex formation between Kp1 and Kp2 proteins followed by a slow rate-limiting unimolecular process, is consistent with the data:

Whether k_2 is the rate constant for electron transfer or for a slow conformation change or hydrolysis of ATP necessary before a rapid electron transfer, cannot be inferred from the data. The dissociation constant for the Kp1Kp2 protein complex must be $K_{D(\text{complex})} < 0.5 \mu\text{M}$ for the reaction to exhibit essentially unimolecular kinetics with $[\text{Kp1}] = [\text{Kp2}] =$

$5 \mu\text{M}$. This is consistent with a value of $K_{D(\text{complex})} = 0.05 \mu\text{M}$ obtained from the non-linear dependence of acetylene reduction activity at low protein concentrations, i.e. 'dilution effect data' (R. N. F. Thorneley, unpublished observation). It also follows that complex formation must occur with $k_{+1} > 1 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$. If a value of $k_{+1} = 1 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$ is assumed for the diffusion-controlled limit for a bimolecular reaction involving two large proteins, then $k_{-1} < 50 \text{ s}^{-1}$. A value of $k_2 = 2.0 \times 10^2 \text{ s}^{-1}$ was extrapolated from the dependence of τ on MgATP concentration (see below).

The uniform increase in amplitude on increasing $[\text{Kp1}]/[\text{Kp2}]$ protein ratios from 1:1 to 1:3 cannot have been due to the displacement of an equilibrium involving the two proteins, since no change in amplitude was observed on increasing the Kp1 protein concentration such that $[\text{Kp1}]/[\text{Kp2}]$ was 4:1. Also the insensitivity of τ to protein concentration requires tight binding of Kp1 and Kp2 proteins before electron transfer. It is possible that Kp2 protein with a specific activity of 1100 nmol of acetylene produced/min per mg of protein is only one-third active and that the 1:3 ratio is in fact a 1:1 ratio of active proteins. Specific activities for Cp2 protein of 3100 nmol of acetylene produced/min per mg of protein (Tso *et al.*, 1972) and 2700 nmol of acetylene produced/min per mg of protein (Nakos & Mortenson, 1971), for Av2 protein of 1815 nmol of acetylene produced/min per mg of protein (Shah & Brill, 1973) and for Ac2 protein of 2000 nmol of acetylene produced/min per mg of protein (M. G. Yates & K. Planqué, personal communication) have been reported. However, since Kp2, Cp2 and Ac2 proteins contain four Fe atoms, four S^{2-} atoms and for each species two identical peptides (Av2 protein has not been characterized), the range of specific activities probably reflects species differences. This is supported by the different molecular weights: Kp2, 67000 (Eady *et al.*, 1972); Cp2, 56000 (Tso, 1974); Ac2, 64000 (M. G. Yates & K. Planqué, personal communication). Eady (1973) observed 1:1 complex formation between Kp2 protein of specific activity 890 nmol of acetylene produced/min per mg of protein and Kp1 protein of specific activity 1130 nmol of acetylene produced/min per mg of protein and did not detect any protein incapable of complex formation.

A feasible explanation for the increase in amplitude is that the first equivalent of Kp2 protein is slowly oxidized ($k_2 = 2.0 \times 10^2 \text{ s}^{-1}$) and that up to two more equivalents of Kp2 protein are then rapidly oxidized ($k_4 > 1 \times 10^3 \text{ s}^{-1}$). Three consecutive oxidations of Kp2 protein with the same rate constant would have given an observed τ of about 2.3 times that for the one equivalent process (Olson *et al.*, 1974). Three concurrent oxidations of 3 mol of Kp2 protein by 1 mol of Kp1 protein would seem to be unlikely, since

this would involve a Kp1/Kp2 protein complex of 1:3. No such complex was observed by Eady (1973) in the ultracentrifuge. The mediation of electron transfer from free Kp2 protein to Kp1 protein by Kp2 protein bound to Kp1 protein in a complex is supported by steady-state-activity titration data (Eady *et al.*, 1972). The fact that maximum activity for Kp1 protein occurs at a Kp1/Kp2 protein ratio of 1:10 in the steady state and not a 1:3 ratio, could be associated with the rate of reduction of Kp2 protein by $\text{Na}_2\text{S}_2\text{O}_4$ and may be the optimum ratio required to maintain an optimum steady-state concentration of reduced Kp2 protein.

Cp2 protein is a two-electron donor (Walker & Mortenson, 1973) and by analogy the oxidation of 3 mol of Kp2 protein by Kp1 protein would result in the transfer of six electrons. The reduction of dinitrogen to NH_3 requires six electrons, which suggests that the two Mo atoms, if they do bind the dinitrogen molecule, interact with dinitrogen in a concerted mode, possibly as in the binuclear dinitrogen complexes discussed by Chatt & Leigh (1972). A less-attractive alternative is that a single Mo atom is involved in substrate reduction and that a 'flip-flop' mechanism operates in which each molybdenum is used alternately for turnover. A third possibility is that the Kp1 protein is only half active even though it has close to 2 Mo atoms/mol; precedents for this situation are xanthine oxidase (Bray, 1975) and succinate dehydrogenase (Beinert *et al.*, 1974), which can exist in high- and low-activity forms containing the same amount of iron and molybdenum.

The dependence of the rate of electron transfer on MgATP concentration can be explained by assuming a rapid reversible binding of MgATP followed by a slow, essentially irreversible electron transfer:

Then with $k_{-3}/k_{+3} = K_{D(\text{MgATP})}$:

$$\tau = \frac{1}{K_{D(\text{MgATP})}[\text{MgATP}]} + \frac{1}{k_2}$$

This rate law was obeyed (Fig. 3) for [MgATP] of 0.063–0.66 mM. At [MgATP] of 1.4 and 4.1 mM, τ remained constant at 8 ms, instead of falling to the extrapolated values of 6 and 5 ms respectively. This may be the onset of the inhibition by MgATP reported by Ljones (1973) for Cp nitrogenase which occurred at high (>10 mM) MgATP concentrations. No inhibition of acetylene reduction by [MgATP] up to 1.5 mM for Kp nitrogenase at 15°C was observed (Thorneley & Willison, 1974). Since electron transfer from Kp2 protein to Kp1 protein is not rate limiting

in turnover, a high degree of inhibition of this reaction may be necessary before it would be reflected in acetylene reduction data. Extrapolation of the linear portion of Fig. 3 gives $K_{D(\text{MgATP})} = 0.4 \text{ mM}$ and $k_2 = 2 \times 10^2 \text{ s}^{-1}$. Smith *et al.* (1973) used a change in e.p.r. signal of Kp2 protein resulting from MgATP binding to determine a dissociation constant $K_{D(\text{MgATP})} = 0.4 \text{ mM}$. This value has been criticised by Thorneley & Willison (1974) since no account was taken of the formation of inactive Mg_2ATP at high Mg^{2+} concentrations. Steady-state data for acetylene reduction gave an apparent $K_{m(\text{MgATP})} = 0.4 \text{ mM}$ at 15°C (Thorneley & Willison, 1974). Thus data from three techniques indicate $K_{m(\text{MgATP})} = K_{s(\text{MgATP})} = 0.4 \text{ mM}$ for Kp nitrogenase. In order for the binding of MgATP to Kp2 protein or to a Kp1Kp2 protein complex to be a rapid preequilibrium before electron transfer, $k_{-3} \gg k_2 = 2 \times 10^2 \text{ s}^{-1}$ i.e. $k_{-3} > 1 \times 10^3 \text{ s}^{-1}$ and with $k_{-3}/k_{+3} = 0.4 \text{ mM}$, then $k_{+3} > 2.5 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$. The coincidence of the dissociation constant calculated in the above analysis, the value from e.p.r. studies and the apparent K_m from steady-state data strongly favour the above mechanism. The lower limit of $k_{+3} > 2.5 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ for the binding of MgATP to Kp2 protein is consistent with the view of Gutfreund (1972) that rates of 1×10^7 – $1 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$ are typical of enzyme-substrate binding processes.

Tso & Burris (1973) used a gel-equilibration method to determine a $K_{D(\text{MgATP})} = 17 \mu\text{M}$ for Cp2 protein. E.p.r. data (Zumft *et al.*, 1974) supported this value, although both the gel equilibration and e.p.r. data have been criticised by Eady & Smith (1974). The difference in dissociation constant for MgATP binding to Cp2 and Kp2 protein is most likely another reflexion of species differences, as was discussed above for specific activities.

Zumft *et al.* (1974) have shown that dye-oxidized Cp2 protein is rapidly ($t_{\frac{1}{2}} < 5 \text{ ms}$) reduced by 10 mM $\text{Na}_2\text{S}_2\text{O}_4$. Since Kp2 protein when oxidized by Kp1 protein in the presence of MgATP is not rapidly reduced, it must be concluded that a slow process must occur before reduction of oxidized Kp2 protein during turnover. Since 2 molar equivalents of ATP are hydrolysed for every electron transferred to substrate (Eady & Smith, 1974), hydrolysis of ATP or release of ADP from Kp2 protein may limit the rate reduction of oxidized Kp2 protein.

Zumft *et al.* (1974) have reported that complex formation between MgATP and Cp2 protein is slow ($t_{\frac{1}{2}}$ approx. 1 s) and much faster in the presence of Cp1 protein ($t_{\frac{1}{2}}$ approx. 10–40 ms). No conclusions about the rate of binding of MgATP to Cp2 protein can be made from this data, since no concentration-dependence of the rate was reported. However, with a $K_{D(\text{MgATP})} = 17 \mu\text{M}$ for Cp2 protein (Tso & Burris, 1973) these half-lives probably refer to rate-limiting conformation changes or electron transfers and not to binding processes.

Table 3. Summary of rate and equilibrium constants

Complex formation between Kp1 and Kp2 proteins (possibly induced by MgATP):

where

$$k_{+1} > 1 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1} \text{ and } k_{-1} < 50 \text{ s}^{-1}. K_{D(\text{complex})} < 0.5 \mu\text{M}$$

Electron transfer or rate-limiting reactions before rapid electron transfer:

where

$$k_2 = 2.0 \times 10^2 \text{ s}^{-1}; k_4 > 1 \times 10^3 \text{ s}^{-1} \text{ (2nd and 3rd mol of Kp2 oxidized).}$$

MgATP binding to Kp2 protein to the Kp1Kp2 protein complex:

where

$$k_{+3} > 2.5 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1} \text{ and } k_{-3} > 1 \times 10^3 \text{ s}^{-1}. K_D(\text{MgATP}) = 0.4 \text{ mM}$$

In this study, since premixing Kp2 protein with MgATP did not change the rate of oxidation of Kp2, the conformation change observed by e.p.r. (Smith *et al.*, 1973) and by thiol-group reactivity (Thorneley & Eady, 1973) on mixing Kp2 protein with MgATP cannot be limiting the rate of electron transfer. Zumft *et al.* (1974) have shown that on binding MgATP, Kp2 protein undergoes a conformation change which shifts the redox potential from -290 to -400 mV .

A summary of the rate and equilibrium constants calculated and estimated in this study are given in Table 3. Since only lower limits can be given for the bimolecular rate constants for complex formation between Kp1 and Kp2 proteins and between Kp2 protein (or Kp1Kp2 protein complex) and MgATP, it is not possible to conclude the order of assembly of the Kp1Kp2-MgATP complex and in particular whether complex formation between Kp1 and Kp2 proteins is induced by MgATP.

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