Electron-transfer studies involving flavodoxin and a natural redox partner, the iron protein of nitrogenase

Conformational constraints on protein–protein interactions and the kinetics of electron transfer within the protein complex

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The kinetics of electron-transfer reactions involving flavodoxins from *Klebsiella pneumoniae* (KpFld), *Azotobacter chroococcum* (AcFld), *Anacystis nidulans* (AnFld) and *Megasphaera elsdenii* (MeFld), the free, MgADP-bound and MgATP-bound forms of the Fe protein component of nitrogenase from *K. pneumoniae* [Kp2, Kp2(MgADP)₂ and Kp2(MgATP)₂] and Na₂S₂O₄ were studied by stopped-flow spectrophotometry. Kinetic evidence was obtained for the formation of binary protein complexes involving KpFldSQ (semiquinone) with either Kp2(MgADP)₂ ($K_D = 49 \mu\text{M}$) or Kp2(MgATP)₂ ($K_D = 13 \mu\text{M}$) but not with Kp2 ($K_D > 730 \mu\text{M}$). The binding of 2MgATP or 2MgADP to Kp2 therefore not only shifts the midpoint potential (E_m) of the [4Fe-4S] centre from -200 mV to -320 mV or -350 mV respectively but also changes the affinity of Kp2 for KpFldSQ. Thermodynamically unfavourable electron transfer from Kp2(MgADP)₂ and Kp2(MgATP)₂ to KpFldSQ occurs within the protein complexes with $k = 1.2 \text{ s}^{-1}$ ($\Delta E = -72 \text{ mV}$) and 0.5 s^{-1} ($\Delta E = -120 \text{ mV}$) respectively. Although AcFldSQ is reduced by Kp2, Kp2(MgADP)₂ and Kp2(MgATP)₂ ($k = 8 \times 10^3$, 2.4×10^3 and $9 \times 10^2 \text{ M}^{-1} \cdot \text{s}^{-1}$ respectively), protein–complex formation is weak in each case ($K_D > 700 \mu\text{M}$). Electron transfer in the physiologically important and thermodynamically favourable direction from Kp2FldHQ (hydroquinone) and AcFldHQ to Kp2_{ox}(MgADP)₂ (the state of Kp2 that accepts electrons from FldHQ in the catalytic cycle of nitrogenase) is rapid ($k > 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$). The second-order rate constants for the reduction of KpFldSQ, AcFldSQ, AnFldSQ and MeFldSQ by SO₂^{•−} (active reductant formed by the predissociation of S₂O₄^{2−} ion) exhibited the linear free-energy relationship predicted by the Marcus theory of electron transfer.

INTRODUCTION

Flavodoxins are small (M_r approx. 20000) FMN-containing monomeric proteins that can serve as electron donors to nitrogenase in the facultative anaerobe *Klebsiella pneumoniae* (oxytoca) (Hill & Kavanagh, 1980; Nieva-Gomez *et al.*, 1980; Shah *et al.*, 1983) and the obligate aerobes *Azotobacter chroococcum* (Yates, 1972) and *Azotobacter vinelandii* (Benemann *et al.*, 1969, 1971; van Lin & Bothe, 1972; Klugkist *et al.*, 1985, 1986). Deistung *et al.* (1985) and Deistung & Thorneley (1986) reviewed the role of flavodoxins in N₂ fixation and described the purification and characterization of flavodoxins from *K. pneumoniae* (KpFld) and *A. chroococcum* (AcFld). In the case of KpFld, this involved the cloning of its gene (*nifF*) and the construction of a strain of *K. pneumoniae* that synthesized and retained an enhanced concentration of active flavodoxin. They also determined the midpoint potentials (E_m) for the semiquinone–hydroquinone couple for KpFld [-422 mV (NHE)] and AcFld (-522 mV), and discussed the significance of

these values with respect to the electron-transport pathway(s) to nitrogenase in these organisms.

The Mo-containing nitrogenase from *K. pneumoniae* comprises two proteins, the tetrameric MoFe protein (Kp1) (M_r 218000) and the dimeric Fe protein (Kp2) (M_r 67000). Kp2 contains a single redox-active [4Fe-4S] cluster and two sites that bind MgATP or MgADP (Ashby & Thorneley, 1987). Kp2(MgATP)₂ transfers one electron to Kp1 in a reaction that is coupled to the hydrolysis of 2MgATP to 2MgADP + 2P_i (Eady *et al.*, 1978; Hageman *et al.*, 1980). Kp2_{ox}(MgADP)₂, after dissociation from reduced Kp1, is reduced in the catalytic cycle by SO₂^{•−} (formed by the predissociation of S₂O₄^{2−} ion). Replacement of 2MgADP by 2MgATP subsequently occurs on the reduced form of Kp2 (Ashby & Thorneley, 1987). This one-electron Fe protein cycle is repeated eight times in order for the MoFe protein to effect the reduction of N₂ to 2NH₃, and concomitant stoichiometric evolution of H₂ (Thorneley & Lowe, 1983, 1984a,b; Lowe & Thorneley, 1984a,b).

Kinetic investigations of electron transfer from flavo-

Abbreviations used: the nitrogenase components of the various organisms are denoted by a capital letter indicating the genus and a lower-case indicating the species; the number 1 indicates the MoFe-containing protein and the number 2 the Fe-containing protein: Kp, *Klebsiella pneumoniae* (oxytoca); Av, *Azotobacter vinelandii*; Ac, *Azotobacter chroococcum*; Cp, *Clostridium pasteurianum*; An, *Anacystis nidulans*; Me, *Megasphaera elsdenii*. The subscript ox. (e.g. Kp2_{ox}) indicates the oxidized species. Flavodoxins from these organisms are denoted by the appropriate genus/species abbreviation as a prefix to Fld, with HQ, SQ or Q indicating hydroquinone, semiquinone or quinone state of reduction respectively (e.g. KpFldSQ). HiPIP, high-potential iron protein from *Chromatium vinosum*. DQ, diquat (1,1'-ethylene-2,2'-bipyridylum dibromide). NHE, normal hydrogen electrode.

Scheme 1. Electron-transfer reactions involving Kp2 with flavodoxin in the presence of Na₂S₂O₄

Kp2* represents Kp2, Kp2(MgADP)₂ or Kp2(MgATP)₂. Fld represents KpFld or AcFld.

doxin to purified nitrogenase have been restricted to steady-state C₂H₂-reduction assays in which flavodoxin either supplemented or replaced S₂O₄²⁻ ion at substrate concentrations (Yates, 1972; Scherings *et al.*, 1977; Hageman & Burris, 1978; Klugkist *et al.*, 1985). These studies showed that, under certain conditions of limiting S₂O₄²⁻ concentration and a MoFe/Fe-protein ratio of > 1:1, flavodoxin could enhance the steady-state rate of C₂H₂ reduction. Lowe & Thorneley (1984b) rationalized these observations and recognized the importance of measuring directly the rate of electron transfer from KpFldHQ to Kp2_{ox}, and the modulation of the kinetics of this reaction by MgATP, MgADP and Kp1.

The present paper describes the kinetics of reduction of KpFldSQ and AcFLdSQ by SO₂^{·-} and the catalysis of these reactions by Kp2, Kp2(MgADP)₂ and Kp2(MgATP)₂. The kinetics of oxidation of KpFldHQ and AcFldHQ by HSO₃⁻/SO₃²⁻ and by Kp2_{ox}(MgADP)₂ are also presented. These reactions are summarized in Scheme 1 and are of interest not only in the context of N₂ fixation but also in terms of general mechanisms of electron transfer between proteins. Very few electron-transfer reactions between physiologically interacting proteins have been studied, and none of these involves a flavodoxin [see Marcus & Sutin (1985) and Mayo *et al.* (1986) for reviews]. The KpFld-Kp2 system is thus unique in that the characterization of *nifF* (KpFld) mutant strains of *K. pneumoniae* enabled unambiguous pairing of a flavodoxin with its redox partner *in vivo* to be made [see Deistung *et al.* (1985) for a review of the genetic evidence].

MATERIALS AND METHODS

Flavodoxin preparation

KpFld was purified as previously described (Deistung *et al.*, 1985). The procedure of Yates (1972) with the addition of two preparative gel-electrophoresis steps

AcFldHQ were prepared by addition of excess Na₂S₂O₄, followed by gel filtration (as described above). FldHQ and FldSQ solutions were standardized spectrophotometrically by using previously reported techniques and absorption coefficients (Deistung & Thorneley, 1986).

Preparation and oxidation of Kp2 protein

Kp2 protein was purified, characterized and oxidized as previously described (Thorneley & Lowe, 1983). Reduced Kp2 in the absence of Na₂S₂O₄ was prepared by anaerobic gel filtration on Sephadex G-25 equilibrated with O₂-free 25 mM-Hepes buffer (pH 7.4)/10 mM-MgCl₂.

Strict anoxia was ensured by preparing all solutions and performing stopped-flow spectrophotometry inside a glove-box through which N₂ gas containing less than 1 p.p.m. of O₂ was continuously circulated (Thorneley & Lowe, 1983). Kinetic data analysis and curve fitting were done as previously described (Thorneley & Lowe, 1983; Ashby & Thorneley, 1987).

All biochemicals were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.) and salts from BDH Chemicals (Poole, Dorset, U.K.). Diquat was a gift from Dr. T. Hawkes of I.C.I. Plant Protection Division. Na₂S₂O₄ (80–85% purity) solutions were standardized spectrophotometrically by titration with K₃FeCN₆. All reactions were studied in a medium containing 25 mM-Hepes buffer (pH 7.4)/10 mM-MgCl₂ at 23 °C.

RESULTS AND DISCUSSION

Reduction of KpFldSQ, AcFldSQ, AnFldSQ and MeFldSQ by Na₂S₂O₄ (k₊₅, Scheme 1)

The dissociation reaction of S₂O₄²⁻ ion (eqn. 1) complicates the kinetic analysis of reactions in which it acts as a reductant:

(Deistung *et al.*, 1985) was used to purify AcFld. Purified MeFld and AnFld were gifts from Dr. H. Haaker and Professor M. Ludwig respectively.

Flavodoxins in the semiquinone state were prepared by stoichiometric reduction of the oxidized forms with Na₂S₂O₄. Na₂SO₃ was then removed by anaerobic gel filtration on Sephadex G-25 equilibrated with 25 mM-Hepes buffer (pH 7.4)/10 mM-MgCl₂. KpFldHQ and

Although both S₂O₄²⁻ and SO₂^{·-} have been shown to be active species in the reduction of proteins (Lambeth & Palmer, 1973; Creutz & Sutin, 1973; Bradic & Wilkins, 1984), in the present system only SO₂^{·-} functions and eqn. (3) is the appropriate rate expression under conditions where the back reaction (k₋₅) can be ignored [see Ashby & Thorneley (1987) for a derivation]:

$$d[\text{FldSQ}]/dt = K^{1/2} k_{+5} [\text{S}_2\text{O}_4^{2-}]^{1/2} [\text{FldSQ}] \quad (3)$$

Fig. 1. Kinetics of reduction of various flavodoxin semiquinones to the hydroquinone state by $\text{Na}_2\text{S}_2\text{O}_4$ (k_{+5}) at pH 7.4 at 23 °C

The data were obtained by stopped-flow spectrophotometry at 580 nm with $[\text{FldSQ}]$ 5 μM and $[\text{Na}_2\text{S}_2\text{O}_4]$ varied from 0.9 to 22 mM (mixed concentrations). The observed first-order rate constant (k_{obs}) is plotted as a function of $[\text{Na}_2\text{S}_2\text{O}_4]$ for MeFldSQ (∇), KpFldSQ ($\bullet$), AnFldSQ ($\blacktriangle$) and AcFldSQ ($\blacklozenge$). The linear dependences are consistent with $\text{SO}_2^{\cdot-}$ being the active reductant. The slopes of the lines and eqn. (3) were used to calculate the values of k_{+5} given in Table 1.

where $K = 1.4$ mM at 23 °C (Thorneley & Lowe, 1983). The reduction of KpFldSQ, AcFldSQ, AnFldSQ and MeFldSQ was monitored at 580 nm in the stopped-flow spectrophotometer. Single-exponential absorbance-time curves yielded first-order rate constants (k_{obs}) that exhibited a linear dependence on $[\text{S}_2\text{O}_4^{2-}]^{1/2}$ (Fig. 1). Eqn. (3) was used to calculate the values of k_{+5} given in Table 1. In each case complete reduction to the FldHQ occurred, as judged by the amplitudes of the absorbance-time curves, which agreed within $\pm 5\%$ with those calculated from previously determined absorption

coefficients for the SQ and HQ forms (Deistung & Thorneley, 1986). Therefore under these conditions the back reaction (k_{-5}) can be neglected.

Limitations on the amount of purified AnFld and MeFld available allowed the determination of k_{+5} but no other rate constants for these flavodoxins. The data in Table 1 show a linear correlation (coefficient 0.996) between $\log k_{+5}$ and the value of E_m for the four flavodoxins (Fig. 2). There is some uncertainty in the literature value of E_m for AnFld. The value of -470 mV (van Lin & Bothe, 1972) falls closest to the line through the other data points. Sykes & Rogers (1984), who reported an E_m of -414 mV, admit to difficulties in obtaining complete reduction to the hydroquinone state, possibly through O_2 leakage. Thus their value may be in error.

The slope of the line in Fig. 2 corresponds to $\log k_{+5}/\log K_5 = 0.42$ [where $K_5 = k_{+5}/k_{-5}$ (eqn. 2) and can be calculated from the E_m values in Table 1]. This suggests that these reactions can be described by Marcus and related theories of electron transfer [see Marcus & Sutin (1985) for a review]. Tsukahara & Wilkins (1985) demonstrated the applicability of Marcus theory to reactions involving $\text{SO}_2^{\cdot-}$ by obtaining a slope of 0.53 for a plot analogous to that shown in Fig. 2 for the reduction of a series of viologen dications (E_m values in the range -250 to -640 mV). Meyer *et al.* (1983) studied electron-transfer reactions between photoreduced flavins and twelve homologous cytochromes and ten homologous high-potential ferredoxins (HiPIPs) and concluded that free-energy relationships (Marcus, 1968) can be used to describe electron-transfer reactions involving proteins. We conclude that $\text{SO}_2^{\cdot-}$ (unlike Kp2 protein; see below) does not discriminate between these flavodoxins and that the relative rates of electron transfer to FMN are determined principally by the free-energy change on reaction (i.e. the E_m of the flavodoxin). This implies that if the composite second-order rate constant $k_{+5} = k_{\text{ET}}/K_D$ (where K_D is the dissociation constant for pre-equilibrium binding of $\text{SO}_2^{\cdot-}$ to flavodoxin and k_{ET} is the first-order rate constant for electron transfer within the

Table 1. Rate constants for the reduction of flavodoxins (SQ) by $\text{SO}_2^{\cdot-}$ (k_{+5}) and oxidation of flavodoxins (HQ) by $\text{HSO}_3^-/\text{SO}_3^{2-}$ (k_{-5}) together with midpoint potentials for the FldSQ/FldHQ couples at 23 °C at pH 7.4

E_m for $\text{SO}_2^{\cdot-}/\text{SO}_3^{2-}$ at pH 7.4 is -707 mV (Mayhew, 1978). Abbreviation: N.D., not determined.

Flavodoxin	Rate constant for reduction of FldSQ (k_{+5}) ($\text{M}^{-1} \cdot \text{s}^{-1}$)	Rate constant for oxidation of FldHQ (k_{-5}) ($\text{M}^{-1} \cdot \text{s}^{-1}$)	E_m (NHE) (mV)
AcFld	$3.2 \times 10^4 \pm 0.1 \times 10^4$	47 ± 2	-522^*
AnFld	$6.1 \times 10^4 \pm 0.1 \times 10^4$	N.D.	$-470^\dagger$ $-447^\ddagger$ $-414^\S$
KpFld	$1.5 \times 10^5 \pm 0.1 \times 10^5$	3.2 ± 0.2	-422^*
MeFld	$3.4 \times 10^5 \pm 0.1 \times 10^5$	N.D.	$-373^\parallel$

* Deistung & Thorneley (1986).

† van Lin & Bothe (1972).

‡ Entsch & Smillie (1972).

§ Sykes & Rogers (1984).

|| Mayhew *et al.* (1969).

Fig. 2. Linear free-energy relationship demonstrating the applicability of the Marcus theory of electron transfer to the kinetics of reduction of flavodoxin semiquinones by $\text{SO}_2^{\cdot-}$ (k_{+5})

The values of $\log k_{+5}$ are plotted against the E_m value for the four flavodoxins studied (data from Table 1): MeFldSQ (∇), KpFldSQ ($\bullet$), AnFldSQ ($\blacktriangle$) and AcFldSQ ($\blacklozenge$). The line is a least-squares best fit with a slope of 0.42 and a correlation coefficient of 0.996. The Marcus theory of electron transfer predicts a slope of 0.5 provided $\Delta G^0 \ll \lambda$, where ΔG^0 is the free energy change on reaction at a given distance and λ is the reorganizational energy before electron transfer. Chemical systems that are considered to obey Marcus theory give slopes of between 0.37 and 0.51 [see Table III of Marcus & Sutin (1985)].

complex) then the values of K_D for all four flavodoxins are similar and that changes in k_{+5} depend only on k_{ET} . The absence of curvature in the lines of Fig. 1 only allows an estimate of $K_D > 30 \mu\text{M}$ (assuming that a 20% deviation from linearity would have been detected at $[\text{S}_2\text{O}_4^{2-}] = 25 \text{ mM}$ when $[\text{SO}_2^{\cdot-}] = 6 \mu\text{M}$). Thus $k_{\text{ET}} > 1 \text{ s}^{-1}$ and $> 10 \text{ s}^{-1}$ for AcFldSQ and KpFldSQ respectively.

Oxidation of KpFldHQ and AcFldHQ by Na_2SO_3 (k_{-5})

The oxidations of KpFldHQ ($7 \mu\text{M}$) and AcFldHQ ($7 \mu\text{M}$) by Na_2SO_3 (100 mM) in the presence of $\text{Na}_2\text{S}_2\text{O}_4$ (0.2 mM) were each monitored at 580 nm in the stopped-flow spectrophotometer. The amplitude of the single-exponential increase in absorbance indicated complete oxidation to the FldSQ form. The presence of $\text{Na}_2\text{S}_2\text{O}_4$ (in the syringe containing the FldHQ) ensured that the potential of the reacted solution was approx. -380 mV , which prevented any further oxidation of the FldSQ to the quinone state (see Deistung & Thorneley, 1986 for E_m values for the FldSQ–FldQ couples). The stopped-flow data yielded $k_{\text{obs}} = 4.7 \pm 0.2$ and $0.32 \pm 0.02 \text{ s}^{-1}$ for AcFldHQ and KpFldHQ oxidation respectively. The existence of protolytic equilibria involving SO_3^{2-} , HSO_3^- and SO_2 (eqn. 2) only allows apparent second-order rate constants (k_{-5}) of $47 \pm 2 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $3.2 \pm 0.2 \text{ M}^{-1} \cdot \text{s}^{-1}$ to be calculated for AcFldHQ and KpFldHQ respectively (Table 1).

The values of k_{+5} and k_{-5} have been used to evaluate the equilibrium constant (K) in eqn. (4):

Since the rate constants k_{+5} and k_{-5} for the two flavodoxins refer to a common reductant ($\text{SO}_2^{\cdot-}$) and

oxidant ($\text{SO}_2/\text{HSO}_3^-/\text{SO}_3^{2-}$), then K is given by eqn. (5):

$$K = k_{+5}^{\text{Kp}} k_{-5}^{\text{Ac}} / k_{+5}^{\text{Ac}} k_{-5}^{\text{Kp}} \quad (5)$$

The values of k_{+5} and k_{-5} (Table 1) yield $K = 70$, which corresponds to a ΔE_m of 108 mV, in good agreement with the value of 100 mV determined from equilibrium data (Deistung & Thorneley, 1986).

Determination of midpoint potentials for Kp2, Kp2(MgADP)₂ and Kp2(MgATP)₂

Midpoint potentials (E_m) were determined by stopped-flow spectrophotometry by monitoring the change in absorbance at 760 nm when reduced (but $\text{S}_2\text{O}_4^{2-}$ -free) Kp2 was mixed with solutions of diquat dication [DQ^{2+} ; $E_m = -350 \text{ mV}$ (NHE), $\Delta\epsilon_{760} = 2.7 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ on reduction to the monocation (Tsukahara & Wilkins, 1985)]. Kp2 exhibits a negligible change in absorbance at 760 nm on oxidation. When Kp2 (122 μM) was mixed with DQ^{2+} (324 μM), the only absorbance change that occurred was within the instrument dead-time (2 ms) and corresponded to the formation of $5 \pm 1 \mu\text{M} \cdot \text{DQ}^+$ (average of three determinations). The Nernst equation together with $E_m = -350 \text{ mV}$ for the DQ^{2+} – DQ^+ couple yields $E_m = -200 \pm 10 \text{ mV}$ for the Kp2/Kp2_{ox} couple.

E_m values for Kp2(MgADP)₂ ($-320 \pm 2 \text{ mV}$) and Kp2(MgADP)₂ ($-350 \pm 5 \text{ mV}$) were determined in a similar manner after the addition of ATP (10 mM) or ADP (10 mM) to the stopped-flow syringe containing the DQ^{2+} . MgATP and MgADP at these concentrations bind and induce conformational changes in Kp2 within approx. 5 ms (Thorneley & Cornish-Bowden, 1977; Ashby & Thorneley, 1987). The kinetics of the approach to equilibrium were complex with either MgATP or MgADP present and have not been analysed in detail. The reactions were biphasic with approx. 90% of the amplitude associated with a fast phase ($\tau < 50 \text{ ms}$) and approx. 10% with a slow reaction ($\tau > 1 \text{ s}$). The total absorbance change was used to calculate the following equilibrium concentrations: Kp2(MgADP)₂ $-32 \mu\text{M}$, Kp2_{ox}(MgADP)₂ $-29 \mu\text{M}$, DQ^{2+} $-35 \mu\text{M}$ and DQ^+ $-29 \mu\text{M}$; Kp2(MgATP)₂ $-37 \mu\text{M}$, Kp2_{ox}(MgATP)₂ $-25 \mu\text{M}$, DQ^{2+} $-48 \mu\text{M}$ and DQ^+ $-25 \mu\text{M}$ (average of three determinations). Decreasing the initial $[\text{DQ}^{2+}] + [\text{DQ}^+]$ to 34 μM (after mixing) produced the expected decrease in the extent of oxidation of Kp2(MgADP)₂ and Kp2(MgATP)₂. E_m values were calculated assuming that the Kp2 was homogeneous with respect to E_m and ability to equilibrate with DQ^{2+} . Ashby & Thorneley (1987) showed that Kp2_{ox} with a similar specific activity to that used in the present studies was homogeneous with respect to stoichiometric reduction by $\text{SO}_2^{\cdot-}$, binding of 2MgADP and ability to undergo an MgADP-induced conformation change. However, it was only 45% competent at MgATP-induced electron transfer to Kp1. In the present studies in both the calculation of E_m values and the kinetic analysis described below the total Kp2 concentration has been used.

The -120 mV and -150 mV shifts in E_m when 2MgATP and 2MgADP bind to Kp2 compare with -140 mV and -170 mV shifts reported for Av2 (Watt, 1985). However, the E_m for free Kp2 (-200 mV) is 110 mV more positive than that of Av2 (Watt, 1985). Bergström *et al.* (1988) determined E_m values of -450 mV for Ac(MgADP)₂ and -463 mV for Ac2*(MgADP)₂ (Ac2* is the Fe protein component of

Fig. 3. Reduction of KpFldSQ and AcFldSQ by $\text{Na}_2\text{S}_2\text{O}_4$ catalysed by Kp2 at pH 7.4 at 23 °C

The reduction of KpFldSQ and AcFldSQ was monitored at 580 nm in the stopped-flow spectrophotometer. Syringe A contained KpFldSQ (11 μM) or AcFldSQ (10 μM). Syringe B contained $\text{Na}_2\text{S}_2\text{O}_4$ (1.8 mM or 0.9 mM) with various concentrations of Kp2 (0 to 300 μM). Both syringes contained Hepes buffer, pH 7.4 (25 mM), and MgCl_2 (10 mM). Under all conditions a single-exponential decrease in absorbance was observed (see Fig. 4) with an amplitude corresponding to complete reduction to yield KpFldHQ and AcFldHQ. Linear plots of k_{obs} versus $[\text{Kp2}]$ are shown for (a) KpFldSQ with $[\text{S}_2\text{O}_4^{2-}]$ 0.9 mM ($\bullet$) and 0.45 mM ($\circ$), and for (b) AcFldSQ with $[\text{S}_2\text{O}_4^{2-}]$ 0.9 mM ($\blacklozenge$). The rate constants given in Table 2 were calculated from the slopes of the lines by using eqn. (6) and the assumptions stated in the text.

the V-containing nitrogenase in *A. chroococcum*). Thus the E_m values for three forms of Kp2 are all approx. 100 mV more positive than those for the corresponding forms of Av2 and at least one form of Ac2. These differences are reflected in the E_m values of the flavodoxins that serve as electron donors to nitrogenase *in vivo*: KpFldHQ/SQ, -422 mV (Deistung & Thorneley, 1986), AvFldHQ/SQ, -500 mV (Klugkist *et al.*, 1986), and AcFldHQ/SQ, -522 mV (Deistung & Thorneley, 1986).

The E_m of $\text{Kp2}(\text{MgADP})_2$ (-350 ± 5 mV) reported above compares with a value of -398 mV determined by e.p.r. spectroscopy after equilibration with KpFldHQ (Deistung & Thorneley, 1986). The difference may be due to (a) the effect of freezing and cooling to 20 K in order to observe the $g = 1.95$ feature of $\text{Kp2}(\text{MgADP})_2$ or (b) a shift in E_m by approx. 50 mV when $\text{Kp2}_{\text{ox}}(\text{MgADP})_2$ or $\text{Kp2}(\text{MgADP})_2$ is bound to KpFldHQ or KpFldSQ respectively (see below).

Electron transfer from Kp2 , $\text{Kp2}(\text{MgADP})_2$ and $\text{Kp2}(\text{MgATP})_2$ to KpFldSQ and AcFldSQ (k_{-2} , k_{+3} and k_{-3})

Electron transfer from Kp2, $\text{Kp2}(\text{MgADP})_2$ and $\text{Kp2}(\text{MgATP})_2$ to KpFldSQ and AcFldSQ is thermodynamically unfavourable and impossible to monitor in

a conventional stopped-flow experiment in which, for example, Kp2 is mixed with KpFldSQ. However, these reactions can be studied in the presence of $\text{Na}_2\text{S}_2\text{O}_4$, which rapidly reduces Kp2_{ox} , $\text{Kp2}_{\text{ox}}(\text{MgADP})_2$ and $\text{Kp2}_{\text{ox}}(\text{MgATP})_2$ (Thorneley & Lowe, 1983; Ashby & Thorneley, 1987). This allows reduction of KpFldSQ or AcFldSQ by Kp2, $\text{Kp2}(\text{MgADP})_2$ or $\text{Kp2}(\text{MgATP})_2$ to compete effectively with the relatively slow, direct reduction of KpFldSQ or AcFldSQ by $\text{Na}_2\text{S}_2\text{O}_4$ (i.e. reactions k_{-3} , k_{-2} and k_{-1} compete effectively with k_{+5} in Scheme 1 because the back reactions k_{+1} , k_{+2} and k_{+3} are suppressed by the rapid removal of Kp2_{ox} by $\text{SO}_2^{\cdot-}$, k_{+4}).

Complete reduction of KpFldSQ and AcFldSQ to the HQ forms by $\text{Na}_2\text{S}_2\text{O}_4$ (0.9 mM) occurred with $k_{\text{obs}} = 0.2$ and 0.04 s^{-1} respectively (Fig. 1). Addition of Kp2 (40–300 μM) to the syringe containing the $\text{Na}_2\text{S}_2\text{O}_4$ (1.8 mM) caused k_{obs} to increase linearly with increasing Kp2 concentration for both flavodoxins (Figs. 3a and 3b). Decreasing $[\text{Na}_2\text{S}_2\text{O}_4]$ from 0.9 to 0.45 mM decreased the ordinate intercept by a factor of $\sqrt{2}$ as required by eqn. (3) but did not change the slope (Fig. 3a). These data are consistent with the rate expression (eqn. 6) discussed below that is based on Scheme 1. The fact that the lines in Fig. 3(a) are parallel allows us to eliminate the possibility that the catalysis by Kp2 is due to FldSQ being reduced more rapidly by $\text{SO}_2^{\cdot-}$ when it is bound to Kp2. If this were the case the slopes of the lines in Fig. 3(a) would have been dependent on $[\text{S}_2\text{O}_4^{2-}]$. Since reduction of Kp2_{ox} by $\text{SO}_2^{\cdot-}$ is rapid [$k_{+4} > 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$ (Ashby & Thorneley, 1987)] the back reactions involving Kp2_{ox} (k_{+1} and k_{+2}) can be neglected because of the low steady-state concentration of Kp2_{ox} . Also, since a single-exponential change in absorbance at 580 nm occurred at all concentrations of Kp2 (with an amplitude corresponding to complete reduction of flavodoxins to the HQ state, k_{-1} must be $\gg k_{-2}$ (Fig. 4). The steady-state approximation together with eqns. (1), (2) and (3) yields eqn. (6) for the rate of reduction of KpFldSQ by $\text{Na}_2\text{S}_2\text{O}_4$ in the presence of Kp2 (Scheme 1):

$$-d[\text{KpFldSQ}]/dt = k_{+5}K_3^{1/2}[\text{S}_2\text{O}_4^{2-}]^{1/2}[\text{KpFldSQ}] + \frac{k_{-2}[\text{Kp2}][\text{KpFldSQ}]}{\frac{(k_{-2} + k_{+3})}{k_{-3}} + [\text{Kp2}]} \quad (6)$$

The first term on the right-hand side of eqn. (6) gives the ordinate intercept of Fig. 3 and the second term is responsible for the dependence of k_{obs} on $[\text{Kp2}]$. If rapid pre-equilibrium binding of Kp2 to KpFldSQ is assumed (i.e. $k_{+3} \gg k_{-2}$) then the term $(k_{-2} + k_{+3})/k_{-3}$ reduces to $k_{+3}/k_{-3} = K_3$, the dissociation constant for the complex between Kp2 and KpFldSQ. The relative magnitudes of K_3 and $[\text{Kp2}]$ determine whether a linear or hyperbolic dependence of k_{obs} on $[\text{Kp2}]$ is observed (see below). The data in Fig. 3 are clearly linear and therefore $K_3 \gg [\text{Kp2}]$ for both flavodoxins. The slope of the lines in Fig. 3 allow the composite apparent second-order rate constants (k_{-2}/K_3) to be calculated (Table 2). Lower limits can be obtained for the values of k_{-2} and K_3 by assuming that curvature would have been detected at the highest concentration of Kp2 if $K_3/5 < [\text{Kp2}]$. The choice of the factor 5 for these data (s.d. $\pm 5\%$), probably represents a conservative estimate of the lower limits for k_{-2} and K_3 (Table 2).

Linear dependences of k_{obs} on $[\text{Kp2}(\text{MgADP})_2]$ and

Fig. 4. Stopped-flow traces showing the Kp2-catalysed reduction of KpFldSQ by $\text{Na}_2\text{S}_2\text{O}_4$ at pH 7.4 at 23 °C

The data were obtained under anaerobic conditions as described in the Materials and methods section. The reactions were monitored at 580 nm, where $\Delta\epsilon = 4.1 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ for the reduction of KpFldSQ. Trace A was obtained when KpFldSQ was reduced by $\text{Na}_2\text{S}_2\text{O}_4$ in the absence of Kp2. Syringe A contained KpFldSQ ($11 \mu\text{M}$). Syringe B contained $\text{Na}_2\text{S}_2\text{O}_4$ (1.8 mM). Trace B was obtained when KpFldSQ was reduced by $\text{Na}_2\text{S}_2\text{O}_4$ in the presence of Kp2. Syringe A contained KpFldSQ ($11 \mu\text{M}$). Syringe B contained $\text{Na}_2\text{S}_2\text{O}_4$ (1.8 mM) and Kp2 ($260 \mu\text{M}$). For both traces both syringes also contained HEPES buffer, pH 7.4 (25 mM), and MgCl_2 (10 mM). The amplitudes of both traces correspond to complete reduction of KpFldSQ to KpFldHQ.

Fig. 5. Reduction of AcFldSQ by $\text{Na}_2\text{S}_2\text{O}_4$ catalysed by $\text{Kp2}(\text{MgADP})_2$ or $\text{Kp2}(\text{MgATP})_2$ at pH 7.4 at 23 °C

The reduction of AcFldSQ was monitored at 580 nm in the stopped-flow spectrophotometer as a single-exponential decrease in absorbance with an amplitude that corresponded to complete reduction to give AcFldHQ. Syringe A contained AcFldSQ ($10 \mu\text{M}$). Syringe B contained $\text{Na}_2\text{S}_2\text{O}_4$ (1.8 mM) with various concentrations of Kp2 (0 to $380 \mu\text{M}$) and either ADP (10 mM) or ATP (10 mM). Both syringes contained HEPES buffer, pH 7.4 (25 mM), and MgCl_2 (10 mM). Plots of k_{obs} versus $[\text{Kp2}(\text{MgADP})_2]$ ($\blacklozenge$) and $[\text{Kp2}(\text{MgATP})_2]$ ($\bullet$) are shown. The rate constants given in Table 2 were calculated from the slopes of the lines by using eqn. (6) and the assumptions stated in the text.

on $[\text{Kp2}(\text{MgATP})_2]$ were obtained with AcFldSQ (Fig. 5) and only the composite apparent second-order rate constant (k_{-2}/K_3) could be precisely calculated (Table 2). However, estimates of the lower limits for k_{-2} and K_3 are given in Table 2. These were obtained by use of an analogous rate expression to that shown in eqn. (6) as discussed in the preceding paragraph.

Hyperbolic dependencies for k_{obs} on $[\text{Kp2}(\text{MgADP})_2]$ and on $[\text{Kp2}(\text{MgATP})_2]$ were obtained only with KpFldSQ (Figs. 6 and 7). The insets to Figs. 6 and 7 show linear plots of $[\text{Kp2}(\text{MgADP or MgATP})_2]k'_{\text{obs}}$ versus $[\text{Kp2}(\text{MgATP or MgADP})_2]$, where k'_{obs} is defined in the legend to Fig. 6. These plots are based on a linear form of eqn. (6) and are analogous to Hanes plots that are commonly used in steady-state kinetic analysis of

enzyme-catalysed reactions. The slopes and intercepts of these graphs were used to calculate the values of K_3 and k_{-2} given in Table 2.

The rate and equilibrium constants given in Table 2 are the first to be measured for a reaction involving a flavodoxin and its naturally occurring electron-transfer partner [see Deistung *et al.* (1985) for the evidence that KpFld is the specific electron donor to nitrogenase *in vivo*]. It was not possible to measure the rates of electron transfer in the physiologically important direction $[\text{KpFldHQ} \text{ to } \text{Kp2}_{\text{ox.}}, \text{Kp2}_{\text{ox.}}(\text{MgADP})_2 \text{ or } \text{Kp2}_{\text{ox.}}(\text{MgATP})_2]$ because these reactions were too fast to follow by stopped-flow spectrophotometry (see below). However, the kinetics of the reverse reactions, which are

Table 2. Rate and equilibrium constants for the reduction of KpFldSQ and AcFldSQ by Kp2, $\text{Kp2}(\text{MgADP})_2$ and $\text{Kp2}(\text{MgATP})_2$ at 23 °C at pH 7.4

E_{m} (NHE) values (in mV) are given in parentheses in the first column.

System	Apparent second-order rate constant (k_{-2}/K_3) ($\text{M}^{-1} \cdot \text{s}^{-1}$)	Protein-protein pre-equilibrium constant (K_3) (μM)	Electron-transfer rate constant (k_{-2}) (s^{-1})	ΔE_{m} (mV)
Kp2 (−200) + KpFldSQ (−422)	9.2×10^3	≥ 730	> 6.7	−222
$\text{Kp2}(\text{MgADP})_2$ (−350) + KpFldSQ (−422)	2.4×10^4	49	1.2	−72
$\text{Kp2}(\text{MgATP})_2$ (−320) + KpFldSQ (−422)	3.8×10^4	13	0.5	−102
Kp2 (−200) + AcFldSQ (−522)	0.8×10^3	≥ 700	> 0.6	−322
$\text{Kp2}(\text{MgADP})_2$ (−350) + AcFldSQ (−522)	2.4×10^3	≥ 1000	> 2.4	−172
$\text{Kp2}(\text{MgATP})_2$ (−320) + AcFldSQ (−522)	0.9×10^3	≥ 1000	> 0.9	−202

Fig. 6. Reduction of KpFldSQ by $\text{Na}_2\text{S}_2\text{O}_4$ catalysed by $\text{Kp2}(\text{MgADP})_2$ at pH 7.4 at 23 °C

The reduction of KpFldSQ was monitored at 580 nm in the stopped-flow spectrophotometer as a single-exponential decrease in absorbance with an amplitude that corresponded to complete reduction to give KpFldHQ. Syringe A contained KpFldSQ (11 μM). Syringe B contained $\text{Na}_2\text{S}_2\text{O}_4$ (1.8 mM) with various concentrations of Kp2 (0 to 300 μM) and ADP (10 mM). Both syringes contained Hepes buffer, pH 7.4 (25 mM), and MgCl_2 (10 mM). A plot of k_{obs} versus $[\text{Kp2}(\text{MgADP})_2]$ is shown consistent with the hyperbolic dependence predicted by eqn. (6) when $k_{+3} \gg k_{-2}$ and $K_3 \sim [\text{Kp2}(\text{MgADP})_2]$. The inset shows a linear dependence of $[\text{Kp2}(\text{MgADP})_2]/k'_{\text{obs}}$ on $[\text{Kp2}(\text{MgADP})_2]$ where $k'_{\text{obs}} = k_{\text{obs}} - (k_{\text{obs}})$ when $[\text{Kp2}(\text{MgADP})_2] = 0$. The intercept and slope of the least-squares best-fit straight line through the data points were used with eqn. (6) to calculate the rate and equilibrium constants given in Table 2. The hyperbola through the data points in the main Figure was drawn by using eqn. (6) and these parameters.

much slower owing to the unfavourable free-energy changes (ΔE_m -322 to -72 mV; Table 2), provide insight not only into the nature of the interaction between flavodoxin and its redox partner but also into the rate of intermolecular electron transfer within the protein complex from the [4Fe-4S] cluster in Kp2 to the FMN in the flavodoxin. The relatively tight binding of KpFldSQ to the MgADP-bound and MgATP-bound conformations of Kp2 ($K_3 = 49$ and 13 μM respectively) but weak binding ($K_3 > 730 \mu\text{M}$) to the nucleotide-free conformation shows that the nucleotide-induced conformational change not only affects the [4Fe-4S] cluster (as reflected in the changes in E_m ; Table 2) but also the 'docking' surface on the protein that interacts with the flavodoxin [see Lowe *et al.* (1985) and Ashby & Thorneley (1987) for reviews of evidence for nucleotide-induced conformational changes in nitrogenase Fe proteins]. We conclude that KpFldSQ exhibits a high degree of specificity for binding to the nucleotide-induced conformations of Kp2. It may be noted that $\text{Kp2}_{\text{ox}}(\text{MgADP})_2$ is the form of Kp2 that is reduced in the catalytic cycle with $\text{Na}_2\text{S}_2\text{O}_4$ as reductant (Thorneley & Lowe, 1983; Lowe & Thorneley, 1984a; Ashby & Thorneley, 1987), and it is presumably this form of Kp2 that interacts with KpFldHQ *in vivo*.

Fig. 7. Reduction of KpFldSQ by $\text{Na}_2\text{S}_2\text{O}_4$ catalysed by $\text{Kp2}(\text{MgATP})_2$ at pH 7.4 at 23 °C

Details were as given in the legend to Fig. 6, except syringe B contained ATP (10 mM) instead of ADP (10 mM).

The weak binding of AcFldSQ to all forms of Kp2 ($K_3 > 700 \mu\text{M}$) indicates important differences at the 'docking' surfaces of KpFldSQ and AcFldSQ. This may be related to the unique positive 'patch' of lysine residues that Drummond (1986) deduced, from DNA sequence data for KpFld (*nifF* gene), to be on the same surface as the FMN-binding site. Neither DNA nor protein sequence data are available for AcFld.

We have assigned k_{-2} to an electron-transfer reaction occurring within the $\text{KpFldSQ-Kp2}(\text{MgADP}$ or $\text{MgATP})_2$ protein complex for the following reasons. A rate-limiting conformational change in KpFldSQ is unlikely since (a) the value of k_{-2} depends on whether the reductant is $\text{Kp2}(\text{MgATP})_2$ or $\text{Kp2}(\text{MgADP})_2$ and (b) the rate constants in Table 1 and Fig. 2 for the reduction of a series of flavodoxin semiquinones by $\text{SO}_2^{\cdot-}$ show a good correlation with E_m values, suggesting that rate-limiting conformation changes do not precede reduction of the FMN. The replacement of 2MgADP by 2MgATP on Kp2 is rapid [$k > 10^2 \text{ s}^{-1}$ (Thorneley & Cornish-Bowden, 1977; Ashby & Thorneley, 1987)]. This suggests that any associated conformation changes in Kp2 also occur too rapidly to limit the rate of electron transfer to FldSQ.

The values of k_{-2} are similar to those for long-range electron-transfer reactions involving ruthenium complexes that are covalently attached to specific histidine residues in various metalloproteins (k_{ET} values between 0.04 and 30 s^{-1}) [Crutchley *et al.* (1985), Mayo *et al.* (1986) and Lieber *et al.* (1987) and references cited therein]. The value of $k_{\text{ET}} = 18 \text{ s}^{-1}$ for electron transfer from $[(\text{NH}_3)_5\text{Ru}^{\text{II}}\text{-His-42}]$ to the [4Fe-4S] centre in oxidized HiPIP ($\Delta E_m = +270 \text{ mV}$) is particularly relevant because of the presence of a [4Fe-4S] centre, albeit at a different oxidation level, in Kp2 protein (Jackman *et al.*, 1988).

The rate of electron transfer between the [4Fe-4S] centre in $\text{Kp2}(\text{MgADP}$ or $\text{MgATP})_2$ and the FMN group in KpFldSQ will depend on (a) the distance apart of these groups, (b) the extent of reorganization in the prosthetic groups and protein structure that is necessary before electron transfer to satisfy Frank-Condon restric-

tions, (c) the free-energy change, (d) the nature of the intervening medium and (e) the relative orientation of the prosthetic groups. Of these factors, for the present, we can only comment on the effect of the free-energy change by comparison of the two values of k_{-2} (Table 2). Marcus theory would not seem to apply in as much as $\Delta(\log k_{-2})/\Delta(\log K) = 0.76$ (where K is the equilibrium constant calculated from the E_m values for the isolated proteins; Table 2). However, a rigorous analysis must await E_m values for the two proteins when complexed to each other.

Electron transfer from KpFldHQ and AcFldHQ to $\text{Kp2}_{\text{ox}}(\text{MgADP})_2$ (k_{+1} , k_{-1} and k_{+2})

In the catalytic cycle of nitrogenase $\text{Kp2}_{\text{ox}}(\text{MgADP})_2$ is the form of Kp2 that is reduced by SO_2^{2-} [*in vitro* (Ashby & Thorneley, 1987)] and therefore presumably by KpFldHQ (*in vivo*). The reductions of $\text{Kp2}_{\text{ox}}(\text{MgADP})_2$ (15 μM) by KpFldHQ (90 μM) and AcFldHQ (22 μM) were too fast to monitor in the stopped-flow apparatus (i.e. $k_2/K_1 > 1 \times 10^6$ and $> 5 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ for KpFldHQ and AcFldHQ respectively). The reactions were monitored at the isobestic points for AcFldHQ/SQ (417 nm) and KpFldHQ/SQ (410 nm) in order to maximize the observed change in absorbance due to the [4Fe-4S] chromophore in $\text{Kp2}_{\text{ox}}(\text{MgADP})_2$ on reduction. These lower limits for k_{+2}/K_1 together with the values of k_{-2}/K_3 (Table 2) can be used to estimate a lower limit for the overall equilibrium constants for the two reductions $K > 40$ and $> 2 \times 10^3$ for the equilibration of KpFldHQ and AcFldHQ with $\text{Kp2}_{\text{ox}}(\text{MgADP})_2$ respectively. These values of K correspond to $\Delta E_m > 100$ and > 200 mV, which are both approx. 30 mV greater than the values calculated from the E_m values for the isolated proteins given in the last column of Table 2. This difference may indicate that the E_m values of either one or both prosthetic groups ([4Fe-4S] and FMN) change when the KpFldHQ– $\text{Kp2}_{\text{ox}}(\text{MgADP})_2$ protein complex forms.

A physiological consequence of the high rate of reduction of $\text{Kp2}_{\text{ox}}(\text{MgADP})_2$ by KpFldHQ and the approx. 100 mV difference in E_m values is that low concentrations of KpFldHQ *in vivo* would ensure that electron transfer to nitrogenase did not limit the rate of N_2 fixation. This is consistent with the difficulties in isolating significant quantities of KpFld from wild-type *K. pneumoniae* (oxytoca) [N.C.I.B. 12204 (Hill & Kavanagh, 1980)] and the necessity for constructing a strain of *K. pneumoniae* (oxytoca) that overproduced this protein (Deistung *et al.*, 1985).

A high rate of reduction of $\text{Kp2}_{\text{ox}}(\text{MgADP})_2$ by KpFldHQ was predicted by Lowe & Thorneley (1984b) on the basis of the reported stimulation of substrate reduction rate by addition of AcFldHQ or AvFldHQ to dithionite- or Fe protein-limited nitrogenase assays (Hageman & Burris, 1978; Klugkist *et al.*, 1985).

Although the results presented above contribute to our understanding of biological N_2 fixation, the greater significance of the $\text{Kp2}(\text{MgATP}$ or $\text{MgADP})_2$ –KpFldSQ system is that the relatively tight binding of the proteins ($K_D = 13$ and 49 μM) and the presence of [4Fe-4S] and FMN groups should enable a detailed spectroscopic investigation of a naturally occurring electron-transfer protein complex.

As in this study, many thermodynamically favourable electron-transfer reactions involving naturally occurring

donor–acceptor protein pairs are too rapid to monitor by rapid-mixing techniques at the protein concentrations necessary to detect a pre-equilibrium complex before electron transfer. The method used in the present study to monitor thermodynamically unfavourable electron-transfer reactions and to obtain kinetic evidence for a protein–protein complex may be applicable to other systems. Such studies should aid our understanding of the factors that determine the specificity and kinetics of biological electron-transfer processes.

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