Biochem. J. (1976) **155**, 137–144
Printed in Great Britain

Nitrogenase of *Azotobacter chroococcum*

KINETICS OF THE REDUCTION OF OXIDIZED IRON-PROTEIN BY SODIUM DITHIONITE

By ROGER N. F. THORNELEY, M. GEOFFREY YATES and DAVID J. LOWE
A.R.C. Unit of Nitrogen Fixation and School of Molecular Sciences, University of Sussex, Brighton BN1 9QJ, U.K.

(*Received* 20 *October* 1975)

The kinetics of the reduction of oxidized Fe-protein of nitrogenase from *Azotobacter chroococcum* by sodium dithionite were studied by stopped-flow and rapid-freezing e.p.r. (electron-paramagnetic-resonance) spectroscopy. The appearance of the $g_{av.} = 1.94$ e.p.r. signal (0.24 electron integrated intensity/mol) was associated with a one-electron reduction by $SO_2^{\bullet -}$ with $k > 10^8 M^{-1} \cdot s^{-1}$ at 23°C. A value of $k = 1.75 s^{-1}$ was obtained for the rate of dissociation of $S_2O_4^{2-}$ into $2SO_2^{\bullet -}$ at 23°C. Further reductions by $SO_2^{\bullet -}$ occurred in three slower phases with rate constants in the range 10^4–$10^6 M^{-1} \cdot s^{-1}$. These latter phases have no corresponding e.p.r. signal changes and are probably associated with enzymically inactive protein. The high rate of reduction by $SO_2^{\bullet -}$ of the Fe-protein alone ($k > 10^8 M^{-1} \cdot s^{-1}$) relative to the rate of oxidation of the Fe-protein in the catalytically active Fe:Mo–Fe protein complex ($k = 2.2 \times 10^2 s^{-1}$) and the observation that in the steady state the Fe-protein is substantially oxidized means that at normal assay concentrations another reaction must limit the rate of reduction of Fe-protein during turnover.

Azotobacter chroococcum nitrogenase consists of two proteins: a dimeric protein (mol.wt. 64000) containing 4 non-haem iron atoms, and a tetrameric protein (mol.wt. 227000) containing 22±2 non-haem iron atoms and 2 molybdenum atoms (Yates & Planqué, 1975). Both proteins, an anaerobic environment, MgATP and a source of electrons, probably flavodoxin *in vivo* (Yates, 1972) or sodium dithionite *in vitro* (Bulen *et al.*, 1965), are required for activity. Previous pre-steady-state studies using stopped-flow spectrophotometry (Thorneley, 1975) and rapid-freezing e.p.r.* spectroscopy (Smith *et al.*, 1972, 1973) on nitrogenase from *Klebsiella pneumoniae* have been concerned with electron transfer from the Fe-protein to the Mo–Fe-protein and the role of MgATP in promoting this reaction. Steady-state data for acetylene reduction with nitrogenases from *A. chroococcum* (M. G. Yates, unpublished work) and *K. pneumoniae* (Eady *et al.*, 1972), in which the ratio of the component proteins was varied (i.e. activity titration data), showed that maximal activity was obtained with about a 20-fold excess of Fe-protein. Thorneley (1975) has suggested that this may be a consequence of the slow reduction of Fe-protein by dithionite or a slow reaction (release of ADP or release of bound Fe-protein from a Fe-protein–Mo–Fe-protein complex) before reduction. Consistent with this, Smith *et al.* (1973) showed that the e.p.r. signal of the Fe-protein is substantially diminished in the steady state, which indicates that a proportion of the Fe-protein is oxidized. Hence the kinetics of the reaction of oxidized Fe-protein with $S_2O_4^{2-}$ are of considerable interest. The Fe-protein from *A. chroococcum* has been oxidized with phenazine methosulphate with only about a 20% loss in catalytic activity (acetylene-reduction assay). The changes in absorption spectrum at 425 nm and the appearance of the characteristic $g_{av.} = 1.94$e.p.r signal on reduction of the protein have enabled a detailed kinetic analysis by stopped-flow spectrophotometry and rapid-freezing e.p.r. spectroscopy.

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

Materials and Methods

The component proteins of nitrogenase were purified from a low-gum-producing variant of *A. chroococcum* (N.C.I.B. 8003) developed in this laboratory, as previously described (Yates & Planqué, 1975). The Fe-protein, Ac2, had a specific activity of approx. 2000 nmol of acetylene reduced/min per mg of protein when assayed with saturating concentrations of Ac1 protein under the standard assay conditions of Eady *et al.* (1972).

Dithionite ion was removed from Ac2-protein solutions by anaerobic gel filtration by using Sephadex G-25 equilibrated with 25 mM-Tris/HCl buffer, pH 7.4, containing 10 mM-$MgCl_2$ and dithiothreitol (70μg/ml). A column of dimensions 25cm×1cm gave complete resolution of dithionite ion from Ac2 protein for loadings of 2ml containing about 30mg of protein. A hypodermic needle attached to the end of the column facilitated the anaerobic collection of the dithionite-free protein into a sealed bottle, which was continuously flushed with deoxygenated N_2. The dithionite-free Ac2 protein was oxidized by a 10-fold excess of phenazine methosulphate. After 20min, the excess of phenazine methosulphate was removed by anaerobic gel filtration as described above. Ac2-ox protein (oxidized Ac2 protein) was stored in bead form in liquid N_2, and thawed out under N_2 gas when required. Ac2-ox protein had specific activities of 1200–1500nmol of acetylene reduced/min per mg of protein on recovery from the stopped-flow apparatus.

Protein concentration was measured by the Folin–Ciocalteu method (Lowry *et al.*, 1951), by using bovine serum albumin, previously dried for 24h over P_2O_5, as a standard; dry-weight measurements have shown that no correction factor is necessary for *A. chroococcum* nitrogenase proteins (Yates & Planqué, 1975).

Each protein solution contained 25mM-Tris/HCl buffer, pH7.4, 10mM-$MgCl_2$ and 70μg of dithiothreitol/ml. Buffers were deoxygenated by sparging for 12h with N_2 gas, which had been passed through two Dreschel bottles in series containing photoreduced Methyl Viologen prepared as described by Sweetzer (1967). The reaction of electrochemically reduced Methyl Viologen with O_2, as monitored in the anaerobic stopped-flow apparatus (Thorneley, 1974), indicated $[O_2]<1\,\mu$M in the buffer solutions.

All biochemicals were purchased from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey KT2 7BH, U.K., and salts from BDH, Poole, Dorset, U.K. Sodium dithionite was shown to be 93% pure by spectrophotometric titration with $K_3Fe(CN)_6$ (ε 1026 = $M^{-1}\cdot cm^{-1}$ at 420nm).

Stopped-flow experiments were done at 23°C, and monitored at 425nm in a 1cm-light-path cell. Anaerobic dilutions of stock solutions were done with gas-tight syringes into N_2-flushed vessels fitted with rubber serum caps. Modifications to a commercial stopped-flow apparatus (American Instrument Co., Silver Springs, MD, U.S.A.), necessary to improve the temperature control and anaerobicity have been described (Thorneley, 1974). Absorbance changes occurring in times less than 1s were recorded on a Tektronix 549 storage oscilloscope and slower changes on a Bryans 2700 chart recorder.

Rapid-freezing e.p.r. measurements were carried out as described by Bray *et al.* (1973).

Theory

Kinetics involving dithionite ion as a reducing agent are complex owing to equilibrium (1).

$$S_2O_4^{2-} \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} 2SO_2^{\cdot-} \quad (1)$$

Both $S_2O_4^{2-}$ and $SO_2^{\cdot-}$ have been shown to be active species in the reduction of one- and two-electron-acceptor metalloproteins (Lambeth & Palmer, 1973; Creutz & Sutin, 1973).

Consider the reduction of Ac2-ox protein, eqns. (2), (3) and (4).

$$S_2O_4^{2-} + \text{Ac2-ox} \xrightarrow{k_{+1}} S_2O_4^{-} + \text{Ac2} \quad (2)$$

$$S_2O_4^{-} + \text{Ac2-ox} \xrightarrow{k_{+2}} S_2O_4 + \text{Ac2} \quad (3)$$

and/or

$$SO_2^{\cdot-} + \text{Ac2-ox} \xrightarrow{k_{+4}} SO_2 + \text{Ac2} \quad (4)$$

Eqns. (2) and (3) represent consecutive-competitive second-order reactions involving $S_2O_4^{2-}$ as the reductant; eqn. (4) represents a second-order reaction involving $SO_2^{\cdot-}$ as reductant, obtained from a pre-dissociation of $S_2O_4^{2-}$ (eqn. 1). Lambeth & Palmer (1973) concluded that only in certain limiting cases and then only at defined concentrations is the system amenable to experiment. No evidence has been obtained in this study for reactions (2) and (3), therefore only eqns. (1) and (4) need be considered.

In this case the relevant equations are:

$$-\frac{d}{dt}[S_2O_4^{2-}] = k_{+3}[S_2O_4^{2-}] - k_{-3}[SO_2^{\cdot-}]^2 \quad (5)$$

$$\frac{d}{dt}[SO_2^{\cdot-}] = 2k_{+3}[S_2O_4^{2-}] - 2k_{-3}[SO_2^{\cdot-}]^2 - k_{+4}[SO_2^{\cdot-}][\text{Ac2-ox}] \quad (6)$$

$$-\frac{d}{dt}[\text{Ac2-ox}] = k_{+4}[SO_2^{\cdot-}][\text{Ac2-ox}] \quad (7)$$

Application of the steady-state approximation to eqn. (6) [i.e. $d[SO_2^{\cdot-}]/dt = 0$] gives:

$$2k_{+3}[S_2O_4^{2-}] = [SO_2^{\cdot-}](2k_{-3}[SO_2^{\cdot-}] + k_{+4}[\text{Ac2-ox}]) \quad (8)$$

The first of two useful limiting cases of eqn. (8) is when

$$2k_{-3}[SO_2^{\cdot-}] \gg k_{+4}[\text{Ac2-ox}] \quad (9)$$

The k_{+4}[Ac2-ox] term in eqn. (8) can now be neglected and

$$[SO_2^{\cdot-}] = \sqrt{\frac{k_{+3}}{k_{-3}}[S_2O_4^{2-}]} \quad (10)$$

Substitution into eqn. (7) gives

$$-\frac{d}{dt}[\text{Ac2-ox}] = k_{+4}K^{\frac{1}{2}}[S_2O_4^{2-}]^{\frac{1}{2}}[\text{Ac2-ox}] \quad (11)$$

where $K = k_{+3}/k_{-3} = 1.4 \pm 0.4$ nM (Lambeth & Palmer, 1973), i.e. the dissociation constant for dithionite ion. In this study, pseudo-first-order conditions with $[S_2O_4^{2-}]$ in large excess over [Ac2-ox] were always used. Hence a plot of $k_{obs.}$ versus $[S_2O_4^{2-}]^{\frac{1}{2}}$ should be linear, passing through the origin with a slope of $k_{+4}K^{\frac{1}{2}}$. This was found to be the case for the second, third and fourth phases of the reaction.

The second useful case is when

$$k_{+4}[\text{Ac2-ox}] \gg 2k_{-3}[SO_2^{\bullet -}] \quad (12)$$

The $2k_{-3}[SO_2^{\bullet -}]$ term in eqn. (8) can now be neglected and

$$[SO_2^{\bullet -}] = 2k_{+3}[S_2O_4^{2-}]/k_{+4}[\text{Ac2-ox}] \quad (13)$$

Substitution into eqn. (7) gives

$$-\frac{d}{dt}[\text{Ac2-ox}] = 2k_{+3}[S_2O_4^{2-}] \quad (14)$$

Thus the reaction will be zero order in [Ac2-ox] and linear traces of absorbance versus time are to be expected, whose slopes will be proportional to $[S_2O_4^{2-}]$. This is the case for the first phase of the reaction. However, the inequality in eqn. (12) must break down as the reaction proceeds, since [Ac2-ox] $\rightarrow 0$, and the kinetics will change in the latter stages to first-order in [Ac2-ox].

Results

Reduction of Ac2-ox by sodium dithionite

Stopped-flow experiments monitored at 425 nm in which Ac2-ox was reduced with a large excess of dithionite gave tetraphasic absorbance–time curves. The fastest phase, which could only be measured at $[S_2O_4^{2-}] < 1.0$ mM because of the 1–2 ms mixing and dead time of the apparatus, was well resolved from the second, third and fourth phases. The absorbance–time curves for the latter phases, which represented about 30–40% of the total absorbance change (Fig. 1), were treated as concurrent first-order exponential processes. The slowest phase was evaluated first by using a plot of log $E_t - E_\infty$ versus time, where E_t and E_∞ are absorbance at time t and infinity respectively. The next slowest phase was evaluated by subtraction of the last phase from the total signal. This process was continued until all three first-order rate constants had been calculated. At $[S_2O_4^{2-}] =$ 10 mM, the time constants τ were 0.18, 3.1 and 11.5 s respectively for the second, third and fourth phases. These phases had approximately equal amplitudes of about 10–15% each of the total absorbance change. The dependence of the observed first-order rate constant for each phase on dithionite concentration is shown in Figs. 2 and 3. All first-order rate constants were dependent on the square root of the dithionite concentration. This is interpreted as the first limiting case with $SO_2^{\bullet -}$ as the reductant (see the Theory section). Second-order rate constants for these three phases were calculated from eqn. (11) and are given in Table 1. Substitution of these rate constants into eqn. (9) indicated that the necessary

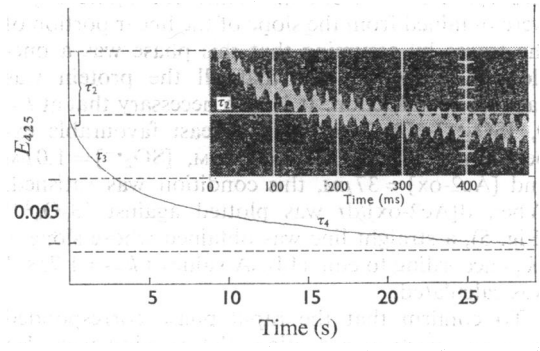

Fig. 1. *Stopped-flow oscillograph and chart-recorder trace for the second, third and fourth phases of the reduction of Ac2-ox protein*

The time-constants were $\tau_2 = 180$ ms, $\tau_3 = 3.1$ s and $\tau_4 = 11.5$ s. τ_1 was too fast to be monitored under these conditions. The graticule of the oscilloscope has been matched with the vertical scale of the chart recorder trace. The difference in noise levels of the two traces is due to the difference in the time-constants for oscilloscope and the chart recorder. Concentrations after mixing were: [Ac2-ox] = 9 μM, $[S_2O_4^{2-}] = 10$ mM, buffer contained 25 mM-Tris/HCl, 10 mM-$MgCl_2$ and 70 μg of dithiothreitol/ml at pH 7.4. Reaction was monitored at 425 nm, and recorded concurrently on the oscilloscope and chart recorder. The lower broken line is the absorbance after 300 s reaction.

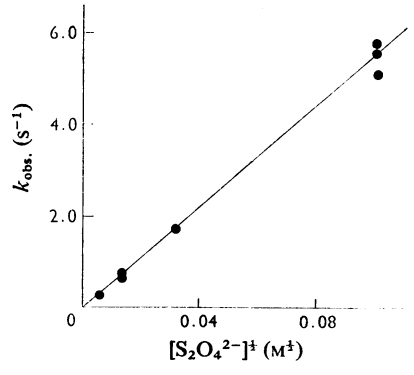

Fig. 2. *Dependence of observed first-order rate constant on $[S_2O_4^{2-}]^{\frac{1}{2}}$ for the second phase of the reduction of Ac2-ox protein*

Slope = 57 $M^{-\frac{1}{2}} \cdot s^{-1}$, which gives $k_{4b} = 1.5 \times 10^6 M^{-1} \cdot s^{-1}$. Concentrations and conditions were as for Fig. 1.

inequality for the rate expression to be valid was complied with over the range of $[S_2O_4^{2-}]$ used.

The fastest phase of the reduction was only observable at $[S_2O_4^{2-}] < 1$ mM and absorbance–time traces were linear initially, becoming exponential in the final stages (Fig. 4*a*). These traces are characteristic of the second limiting case with $SO_2^{\bullet-}$ as reductant (see the Theory section). Values for d[Ac2-ox]/dt were obtained from the slope of the linear portion of the traces by assuming that this phase was a one-electron reduction and that all the protein was capable of reduction. It is also necessary that at $t = 0$, $[SO_2^{\bullet-}] \ll [Ac2\text{-}ox]$. In the least favourable experiment, with $[S_2O_4^{2-}] = 0.8$ mM, $[SO_2^{\bullet-}] = 1.0\,\mu$M and [Ac2-ox] $= 37\,\mu$M, this condition was satisfied. When d[Ac2-ox]/dt was plotted against $[S_2O_4^{2-}]$ (Fig. 5), a straight line was obtained whose slope is $2k_{+3}$ according to eqn. (14). A value of $k_{+3} = 1.75\,s^{-1}$ was calculated.

To confirm that the rapid phase corresponded to a one-electron reduction, the reaction was also monitored at 315nm. At this wavelength the rate and amplitude of $S_2O_4^{2-}$ oxidation can be measured [$S_2O_4^{2-}$ has $\varepsilon_{max.} = 8000\,M^{-1}$ at 315nm, in agreement with the previously reported value (Dixon, 1971)]. No changes in extinction at 315nm were reported by Eady *et al.* (1972) for the oxidation of Kp2 protein, and this was assumed to be true for Ac2 protein. The oxidation products of $S_2O_4^{2-}$ do not absorb at 315nm. A typical oscillograph is shown in Fig. 4(*b*). The amplitude of the rapid phase corresponded to 0.93 ± 0.05 electron equivalent (0.46mol of $S_2O_4^{2-}$)/mol of Ac2-ox protein (average of three determinations). From the slope of the linear portion of the trace in Fig. 4(*b*) and the concentration of $[S_2O_4^{2-}] = 83\,\mu$M, a value of $k_{+3} = 1.6\,s^{-1}$ was calculated for the rate of dissociation of $S_2O_4^{2-}$. The combined amplitudes of the second, third and fourth phases after 7min reaction time corresponded to another 0.9 electron equivalent. Hence, only the first rapid phase corresponded to a one-electron-equivalent reduction.

For zero-order kinetics to be observed in the early stages of the reaction, the inequality in eqn. (12) must be valid. Hence, if we assume that $k_{+3} = 1.2 \times 10^9\,M^{-1} \cdot s^{-1}$ (Lambeth & Palmer, 1973), then a second-order rate constant for the reduction of Ac2-ox protein by $SO_2^{\bullet-}$ of $k_{+4} > 10^8\,M^{-1} \cdot s^{-1}$ is required for the first phase (Table 1).

A rapid-freeze e.p.r. experiment with $[S_2O_4^{2-}] = 10$mM in which the sample was frozen after 20ms reaction showed that the $g_{av.} = 1.94$ signal of the reduced protein was fully developed after 20ms (Fig. 6). Samples frozen at longer times showed no further increase in intensity. It was concluded that the first fast reduction phase with $k > 10^8\,M^{-1} \cdot s^{-1}$ corresponds to the reduction of the e.p.r.-active Fe centre of Ac2 protein and that the second, third and fourth phases observed spectrophotometrically have no corresponding e.p.r. changes.

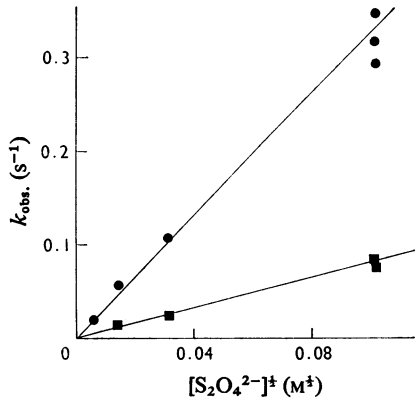

Fig. 3. Dependence of observed first-order rate constants on $[S_2O_4^{2-}]^{\frac{1}{2}}$ for the third (●) and fourth (■) phases of the reduction of Ac2-ox protein

For the third phase the slope $= 3.4\,M^{-\frac{1}{2}} \cdot s^{1-}$ which gives $k_{4c} = 9.1 \times 10^4\,M^{-1} \cdot s^{-1}$. For the fourth phase the slope $= 0.85\,M^{-\frac{1}{2}} \cdot s^{-1}$, which gives $k_{4d} = 2.3 \times 10^4\,M^{-1} \cdot s^{-1}$.

Table 1. *Rate constants for reactions of $SO_2^{\bullet-}$ with non-haem iron proteins and oxygen*

Oxidant	k ($M^{-1} \cdot s^{-1}$)	Comments	Reference
A. chroococcum nitrogenase Fe-protein (Ac2-ox)	$>1.0 \times 10^8$	First phase	This work
	1.5×10^6	Second phase	This work
	9.1×10^4	Third phase	This work
	2.3×10^4	Fourth phase	This work
Spinach ferredoxin	2.3×10^5		Lambeth & Palmer (1973)
C. pasteurianum ferredoxin	5.1×10^4	Initial slopes of first phase	Lambeth & Palmer (1973)
Micrococcus lactilyticus ferredoxin	3.0×10^4		Lambeth & Palmer (1973)
O_2	1.3×10^6		

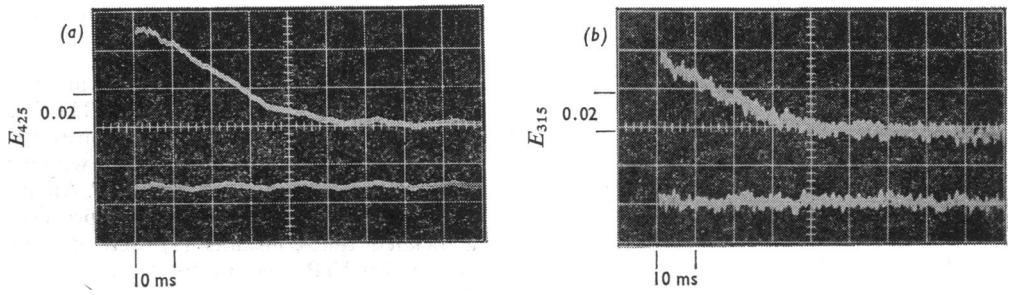

Fig. 4. *Stopped-flow oscillographs for the first phase of the reduction of Ac2-ox protein at low $S_2O_4^{2-}$ concentrations*

(*a*) Reaction was followed at 425nm. Note the linear nature of the trace for about 75% of the initial phase. This is characteristic of a reaction zero order in Ac2-ox concentration. Lower trace represents final absorbance after about 300s. Concentrations after mixing: [Ac2-ox] = 18.5μM, [$S_2O_4^{2-}$] = 0.125mM. Buffer and conditions were as for Fig. 1. (*b*) Reaction was followed at 315nm, where rate of loss of $S_2O_4^{2-}$ can be monitored. Concentrations after mixing: [Ac2-ox] = 11.7μM, [$S_2O_4^{2-}$] = 83μM. Initial phase corresponds to 0.93±0.05 electron equivalent/mol of Ac2-ox protein. Data from this trace are also presented in Fig. 5. Lower trace represents final absorbance after about 300s. Buffer and conditions were as for Fig. 1.

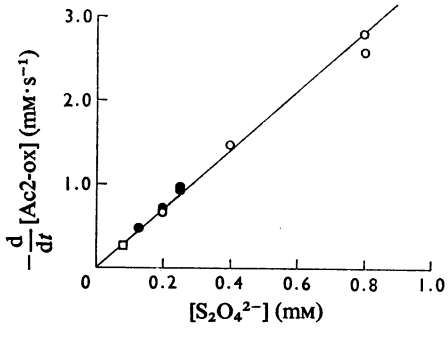

Fig. 5. *Plot of the linear rate of disappearance of Ac2-ox protein obtained from the slopes of traces such as that in Fig. 4(a) versus [$S_2O_4^{2-}$]*

Data at different initial mixed [Ac2-ox] are presented: ●, [Ac2-ox] = 18.5μM; ○, [Ac2-ox] = 37μM; both were followed at 425nm. □, Data point obtained from Fig. 4(*b*) at 315nm by assuming $2d[S_2O_4^{2-}]/dt = d[Ac2\text{-}ox]/dt$. Buffer and conditions were as for Fig. 1. Slope = $2k_{+3} = 3.5s^{-1}$; $k_{+3} = 1.75s^{-1}$.

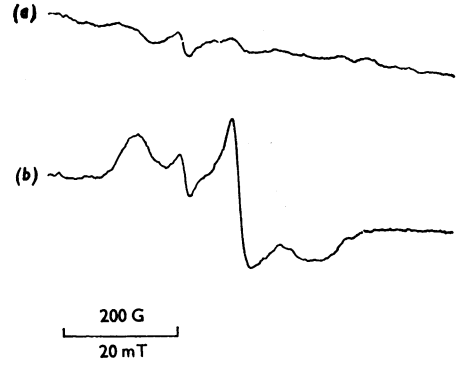

Fig. 6. *E.p.r. spectra showing the reduction of Ac2-ox protein* (37μM) *by* 10mM-$S_2O_4^{2-}$

(*a*) Ac2-ox protein with buffer, no dithionite; (*b*) Ac2-ox protein after 20ms reaction with $S_2O_4^{2-}$. No further increase in intensity of spectrum (*b*) occurred after 30s reaction time. The data were obtained by the rapid-freezing technique. Spectra were recorded at 9GHz, 15°K and 15mW microwave power by using a modulating field of 1.25mT (12.5G).

Reduction of O_2-inactivated Ac2-ox protein by sodium dithionite

The enzymic activity of Ac2-ox protein was destroyed by shaking in air for 10min. Excess of O_2 was removed by flushing with N_2 and then the inactivated protein was reduced by 10mM-$S_2O_4^{2-}$ in the stopped-flow apparatus. The time-constant of the first phase with O_2-damaged protein was now 80ms, compared with less than 1ms with active protein. It was concluded that the rapidly reducible ($k > 10^8 M^{-1}\cdot s^{-1}$) e.p.r.-active centre of Ac2 protein is irreversibly damaged by O_2.

Ac2-ox protein was also prepared in the absence of dithiothreitol. Since dithiothreitol is a reducing agent ($E_0 = -330$mV, pH7.0) and on oxidation has $\Delta\varepsilon \simeq 40 M^{-1}\cdot cm^{-1}$ at 315nm (Cleland, 1964), it was possible that it could interfere with reductions by $SO_2^{\cdot-}$. Ac2-ox protein prepared in the absence of dithiothreitol had a specific activity of 1370nmol of acetylene reduced/min per mg of protein. After reaction in the stopped-flow apparatus, the specific

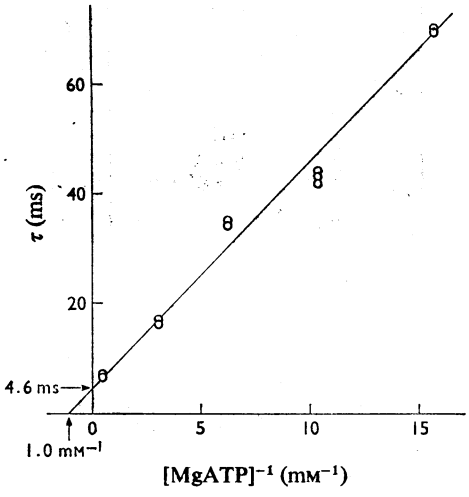

Fig. 7. *Dependence of τ on the reciprocal of MgATP concentration for the oxidation of Ac2 protein* (20 μM) *by Ac1 protein* (7.5 μM)

Reaction was monitored at 425 nm, 23°C; buffer contained 25 mM-Tris/HCl, 10 mM-$MgCl_2$, 10 mM-$Na_2S_2O_4$ and dithiothreitol (70 μg/ml).

activity was only 900 nmol of acetylene reduced/min per mg of protein. However, the amplitude and kinetics of the first phase at 315 nm (loss of $S_2O_4^{2-}$) were identical with those previously observed, but the combined amplitudes of the slower phases were larger (more than 2 electron equivalents after 3 min).

Determination of the rate of dissociation of $S_2O_4^{2-}$

It was important to determine k_{+3} independently of a reaction involving Ac2-ox protein under the same conditions of ionic strength, pH, buffer etc. This was done by following spectrophotometrically the oxidation of $S_2O_4^{2-}$ by O_2 under conditions of $[O_2] > 10[S_2O_4^{2-}]$ at 315 nm, as has been described by Lambeth & Palmer (1973). Under these conditions the observed first-order rate constant can be equated to k_{+3}. When 15 μM-$S_2O_4^{2-}$ was oxidized by 0.65 mM-O_2 in the stopped-flow apparatus, a single exponential decay of $S_2O_4^{2-}$ was observed with $\tau = 550 \pm 5$ ms (average of five determinations), giving $k_{+3} = 1.82 \pm 0.02\ s^{-1}$ at 23°C. Lambeth & Palmer (1973) obtained a value of $k_{+3} = 1.3\ s^{-1}$ at 23°C (calculated from $k_{+3} = 1.7\ s^{-1}$ at 25°C and $\Delta H^{\ddagger} = 100\ kJ \cdot mol^{-1}$). Their value was determined in 0.25 M-NaCl at pH 8.0, and the difference between our value and theirs is probably a consequence of the different ionic strength.

Rate of oxidation of Ac2 protein by Ac1 protein induced by MgATP

A mixture of reduced Ac2 protein (40 μM) and Ac1 protein (15 μM) in 25 mM-Tris/HCl buffer, pH 7.4, containing 10 mM-$Na_2S_2O_4$ and 10 mM-$MgCl_2$ was mixed in the stopped-flow apparatus with a series of solutions containing 5.0–0.2 mM-ATP. An increase in E_{425} occurred in a single exponential process, and the associated time-constants (τ) were determined at various MgATP concentrations (calculated by using $K_{MgATP} = 1.12 \times 10^4\ M^{-1}$ and $K_{Mg_2ATP} = 59\ M^{-1}$; Frey *et al.*, 1972). A plot of τ versus $[MgATP]^{-1}$ (Fig. 7) was linear, with intercepts corresponding to $\tau = 4.6$ ms ($k = 2.2 \times 10^2\ s^{-1}$) for the electron transfer between the two proteins bound in a complex, and an apparent binding constant for MgATP with the protein complex of 1.0 mM. The analogous system with component proteins from *K. pneumoniae* gave values of $k = 2.0 \times 10^2\ s^{-1}$ and $K_{MgATP} = 0.4$ mM (Thorneley, 1975). Rapid-freeze e.p.r. data for the *K. pneumoniae* system (Smith *et al.*, 1973) enabled the rapid absorbance changes seen in the stopped flow to be assigned to an electron-transfer reaction between the two proteins. The similarity of the rate and MgATP-binding constants strongly suggests a common mechanism. A steady-state e.p.r. experiment indicated that Ac2 protein is largely oxidized in the steady state, as has been reported for the *K. pneumoniae* system (Smith *et al.*, 1973). It is concluded that Ac2 protein is rapidly oxidized by Ac1 protein ($k = 2.2 \times 10^2\ s^{-1}$) and that it remains largely oxidized in the steady state.

Discussion

The first phase of the reduction of Ac2-ox protein by $SO_2^{\bullet -}$ is probably the most significant with regard to enzymic activity for the following reasons. (1) It accounts for approx. 50% of the total optical change and corresponds to 0.93 electron equivalent/mol. (2) It correlates with an e.p.r. signal which changes during turnover. (3) It is fast and can be accommodated within the turnover time of the enzyme (500 ms per electron transferred to substrate); the second phase can just be accommodated, but not the third and fourth phases. (4) It is profoundly affected by O_2 damage, whereas the later phases are not. (5) It is strongly inhibited by MgADP, an inhibitor of nitrogenase function (Yates *et al.*, 1975).

A value of $k_{+3} = 1.7\ s^{-1}$ for the dissociation of $S_2O_4^{2-}$, obtained from the kinetics of the first phase at 425 nm, is in good agreement with the values of $k_{+3} = 1.8\ s^{-1}$ and $1.6\ s^{-1}$ obtained from the kinetics of the reaction of O_2 and Ac2-ox protein respectively with $S_2O_4^{2-}$ when followed at 315 nm. This good agreement is important, since it enables the concentration of 'active sites' involved in the rapid phase to

be calculated. The calculations on the data at 315nm only require a value for the extinction coefficient of $S_2O_4^{2-}$ and, for the reaction with Ac2-ox protein, that $\Delta\varepsilon = 0$ for the protein on reduction. However, the calculation for the data obtained by measurement of the rate of reduction of Ac2-ox protein at 425nm depends on an assumed $\Delta\varepsilon$ for the protein in order to convert the slopes of the absorbance–time traces into the units $M \cdot s^{-1}$. Thus the value of k_{+3} will be proportional to the percentage of the protein capable of being reduced in the first phase. The close agreement between the three values of k_{+3} validates the assumption that all the protein contains one site capable of a one-equivalent reduction for the first phase. Further confirmation is that the amplitude corresponds to 0.93 electron equivalent/mol of protein (calculated from the amount of $S_2O_4^{2-}$ oxidized by monitoring the first phase at 315nm). Since the proteins had specific activities of 1200–1500nmol of acetylene reduced/min per mg of protein, considerably less than the maximal activity of 2200nmol of acetylene reduced/min per mg of protein reported by Yates & Planqué (1975), it must be concluded that damage to another more sensitive site on the protein was responsible for loss of catalytic activity, and not the Fe centre, which gives rise to the $g_{av.} = 1.94$ signal, and which is rapidly reduced in a one-equivalent process by $SO_2^{\bullet-}$. This is consistent with the observation that the intensity of the $g_{av.} = 1.94$ signal is not related in a simple manner to activity, i.e. Ac2, Kp2 and Cp2 proteins with activities of 2000, 1000 and 2000 nmol of acetylene reduced/min per mg of protein have integrated e.p.r. signals of 0.17 (Yates & Planqué, 1975), 0.45 (Smith *et al.*, 1973) and 0.8 electron (Orme-Johnson *et al.*, 1972). An integration of 0.2 electron has also been reported for Cp2 protein (Zumft *et al.*, 1973).

Walker & Mortenson (1973) titrated Cp2 protein (mol.wt. 55000, specific activity 2300nmol of acetylene reduced/min per mg of protein) with various oxidizing dyes and obtained values of 1.4–1.7 electron equivalents/mol of Cp2 dimer. However, no time-course for the oxidation, no correlation between oxidizing equivalents added and the intensity of the $g_{av.} = 1.94$e.p.r. signal and no specific activities for Cp2 protein after oxidation were reported. Thus Walker & Mortenson's (1973) data most likely refer to a combination of the rapid e.p.r.-active phase and the slow phases observed in the present study, which do indeed total about 2 electron equivalents per mol of Ac2 protein.

The structure of the Fe centre in the Fe-proteins of nitrogenases from various sources has been investigated by a number of techniques. The 4 Fe atoms per dimer might seem to favour two subunits, each with a two-iron-ferredoxin-type arrangement of the Fe atoms. Although the e.p.r. signal is not inconsistent with this structure (Smith *et al.*, 1973), the Mössbauer spectrum of Kp2 protein (Smith & Lang, 1974) favours a four-iron cluster similar to those in clostridial ferredoxin. Orme-Johnson (1975) has reported that a four-iron centre can be extracted from Cp2 protein by using the thiophenolate ligands developed by Holm (1975). The high rate of reduction by $SO_2^{\bullet-}$ of Ac2-ox protein, $k > 10^8 M^{-1} \cdot s^{-1}$, emphasizes the differences in structure between Ac2 and other non-haem iron proteins, which react at rates at least 100-fold lower (Lambeth & Palmer, 1973) (see Table 1). However, this high reduction rate is not a uniquely fast redox reaction involving a small molecule and a protein. A diffusion-controlled bimolecular rate constant of $2.4 \times 10^9 M^{-1} \cdot s^{-1}$ has been measured for the reaction of $O_2^{\bullet-}$ with superoxide dismutase (Fielden *et al.*, 1974).

The analysis of the slow phase into three concurrent exponential processes is a difficult procedure. Although reasonable dependencies on $[S_2O_4^{2-}]^{\frac{1}{2}}$ were obtained with rate constants in the range 10^4–$10^6 M^{-1} \cdot s^{-1}$ for reduction by $SO_2^{\bullet-}$, it is quite possible that these complex absorbance–time curves represent combinations of concurrent or consecutive bimolecular and unimolecular processes involving conformation changes and/or redistribution of electrons in the protein, in which case the analysis applied above to these phases would not be valid. The time-constants for the third and fourth phases are too slow to be accommodated in the turnover time of the enzyme and may be associated with inactive protein. An intact but catalytically inactive four-iron cluster was identified by Smith & Lang (1974) in O_2-damaged Kp2 protein by using Mössbauer spectroscopy. The second phase, although it corresponds to only about 0.3 electron equivalent, may be significant, since at $[S_2O_4^{2-}] = 1$mM (the apparent K_m for $S_2O_4^{2-}$), the time-constant for reduction approaches the turnover time for the full fixing system. The first phase is far too fast, even at $[S_2O_4^{2-}] = 1$mM, for this reduction to become rate-limiting in turnover. Since this phase is associated with the $g_{av.} = 1.94$ e.p.r. signal, this reaction must be either modified or preceded by a slower reaction involving the oxidized form of Ac2 protein in order for the e.p.r. signal to be diminished during the steady state in the 'full fixing system'. This follows from a comparison of the rate of oxidation of Ac2 protein by Ac1 in the enzymically active complex ($k = 2.2 \times 10^2 s^{-1}$, $\tau = 4.6$ms) with the rate of reduction of Ac2-ox by $SO_2^{\bullet-}$ ($\tau < 1$ms at $[S_2O_4^{2-}] = 10$mM).

The effect of MgATP/ADP and Ac1 protein on the reduction of Ac2-ox by $SO_2^{\bullet-}$ may explain why Ac2 protein remains largely oxidized in the steady state.

D. J. L. thanks the Medical Research Council for support and for the provision of e.p.r. facilities on a programme grant. We also thank Mrs. F. Campbell for technical assistance.

References

Bray, R. C., Lowe, D. J., Capeillère-Blandin, C. & Fielden, E. M. (1973) *Biochem. Soc. Trans.* **1**, 1067–1072

Bulen, W. A., Burns, R. C. & LeCompte, J. R. (1965) *Proc. Natl. Acad. Sci. U.S.A.* **53**, 532–540.

Cleland, W. W. (1964) *Biochemistry* **3**, 480–483

Creutz, C. & Sutin, N. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 1701–1703

Dixon, M. (1971) *Biochim. Biophys. Acta* **226**, 241–245

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

Fielden, E. M., Roberts, P. B., Bray, R. C., Lowe, D. J., Mautner, G. N., Rotilio, G. & Calabrese, L. (1974) *Biochem. J.* **139**, 49–60

Frey, C. M., Banyasz, J. L. & Stuer, J. E. (1972) *J. Am. Chem. Soc.* **94**, 9198–9204

Holm, R. H. (1975) *Endeavour* **34**, 38–43

Lambeth, D. O. & Palmer, G. (1973) *J. Biol. Chem.* **248**, 6095–6103

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Orme-Johnson, W. H. (1975) *Biochem. Soc. Trans.* **3**, 468

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

Smith, B. E. & Lang, G. (1974) *Biochem. J.* **137**, 169–180

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

Smith, B. E., Lowe, D. J. & Bray, R. C. (1973) *Biochem. J.* **135**, 331–341

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

Thorneley, R. N. F. (1974) *Biochim. Biophys. Acta* **333**, 487–496

Thorneley, R. N. F. (1975) *Biochem. J.* **145**, 391–396

Walker, M. & Mortenson, L. E. (1973) *Biochem. Biophys. Res. Commun.* **54**, 669–676

Yates, M. G. (1972) *FEBS Lett.* **27**, 63–67

Yates, M. G. & Planqué, K. (1975) *Eur. J. Biochem.* in the press

Yates, M. G., Thorneley, R. N. F. & Lowe, D. J. (1975) *FEBS Lett.* **60**, 89–92

Zumft, W. G., Palmer, G. & Mortenson, L. E. (1973) *Biochim. Biophys. Acta* **292**, 412–421