NITROGENASE OF *AZOTOBACTER CHROOCOCCUM*: INHIBITION BY ADP OF THE REDUCTION OF OXIDISED Fe PROTEIN BY SODIUM DITHIONITE

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Received 2 October 1975

1. Introduction

Nitrogenase comprises two (non-haem) iron–sulphur proteins, one of which contains molybdenum. Both proteins, an electron donor (ferredoxin, flavodoxin, $Na_2S_2O_4$), Mg-ATP and an anaerobic environment are necessary to reduce substrate; Mg-ADP is an inhibitor. During enzyme turnover electrons are transferred in the direction: donor $\xrightarrow{I}$ Fe protein $\xrightarrow{II}$, Mo–Fe protein $\xrightarrow{III}$ reducible substrate. Spectrophotometric and rapid freeze e.p.r.* studies on the complete enzymically active nitrogenase system indicates that reactions I and III are slow compared with reaction II [1–5].

The nitrogenase system is extremely complex and, therefore, the study of partial reactions involving individual component proteins has contributed to the understanding of the enzyme mechanism. There is good evidence that both ATP and ADP associate with and induce conformational changes in the reduced Fe protein [4,6–8] and that ATP-dependent electron transfer occurs between the reduced Fe protein and oxidised Mo–Fe protein (reaction II,) [1–5]. There have been no detailed kinetic studies on reaction I of the above sequence, possibly because the nitrogenase Fe protein is extremely oxygen sensitive. Zumft et al. [9] reported briefly that rapid freeze e.p.r. spectroscopy showed that oxidised Fe protein from *Clostridium pasteurianum* [Cp2] was rapidly reduced by sodium dithionite. In the present paper we describe kinetic studies by stopped flow spectrophotometry and e.p.r. spectroscopy on the reduction of oxidised Fe protein from *Azotobacter chroococcum* [Ac2-ox] by sodium dithionite (reaction I) and show that there is a very rapid phase of reduction which is not present in oxygen-inactivated Ac2 and which is strongly inhibited by Mg-ADP.

* *Abbreviations and nomenclature:* The component proteins of nitrogenase from various organisms are designated by a capital letter for the genus and a lower case letter for the species, the figure 1 for the Mo–Fe protein and 2 for the Fe protein. Thus in this paper the Fe protein from *A. chroococcum* is Ac2 and its oxidised form is Ac2-ox; that from *K.pneumoniae* is Kp2 and that from *C.pasteurianum* is Cp2. The Mo-Fe protein from *A.chroococcum* is Acl. *Other abbreviations:* e.p.r. = electron paramagnetic resonance.

2. Materials and methods

The growth and harvesting of *A. chroococcum* (NCIB 8003) and the production of enzymically-active oxidised Ac2-ox [10], anaerobic stopped-flow spectrophotometry [11] and rapid-freezing e.p.r. spectroscopy [12] have all been described. Protein concentration was measured by the Folin- Ciocalteau method [13] using bovine serum albumin dried over P_2O_5 as a standard. The specific activities of Ac2 were measured in the presence of saturating levels of Acl and refer to the number of nmoles of acetylene reduced/min/mg of Ac2 protein. Ac2-ox, freed from the oxidant (phenazine methosulphate) by anaerobic gel chromatography on Sephadex G25 was prepared in 25 mM Tris buffer pH 7.4 containing 10 mM $MgCl_2$ and 70 μg of dithiothreitol/ml. In the experiments described in this paper the specific activity of Ac2-ox was 1780 initially. It lost 18% activity during an experimental period (5 h) while stored in a gas-tight syringe at 23°C.

Buffers were flushed for several hours with N_2. The reaction of electrochemically reduced methyl viologen with O_2, as monitored in the anaerobic stopped-flow apparatus [11], indicated $[O_2] < 10^{-6}$ M in the buffers. Stopped-flow spectroscopy was measured in a 1 cm light path at 425 nM. Experiments for analysis by e.p.r. were also performed at 23°C before rapid freezing.

All biochemicals were obtained from Sigma (London) Chemical Co.Ltd. and all salts from British Drug Houses, Poole, Dorset. Sodium dithionite was shown to be 93% pure by spectrophotometric titrations with $K_3Fe(CN)_6$ (ϵ = 1026 at 420 nm).

3. Results

Fig.1 shows time courses for the optical density changes during the reduction of Ac2-ox by 10 mM sodium dithionite. Initially, there is a very fast phase ($\tau < 1$ msec: compare the optical density denoted by the trace in fig.1a with that of the start of the trace in fig.1b) followed by two or more slower phases ($\tau > 100$ msec). This fast phase corresponds to the appearance of the characteristic e.p.r. signal of reduced nitrogenase Fe protein when Ac2-ox was reduced by sodium dithionite (fig.2 traces a and b). The integration (0.24 electron) of the e.p.r. signal developed after 20 msec was within 10% of the integration of the e.p.r. signal developed after 30 sec exposure to sodium dithionite (table 1). Thus there was no e.p.r. signal change corresponding to the slow phases, which account for approx. 40% of the total absorbance change on reduction. The appearance of this characteristic e.p.r. signal is associated with enzymically active nitrogenase Fe protein inasmuch as it changes during the enzyme turnover and is modified by ATP or ADP [4,10]. It did not appear when oxygen-inactivated Ac2 was reduced by sodium dithionite. Fig.3 shows the corresponding absence of a fast initial phase of reduction upon reacting oxygen-inactivated Ac2 with sodium dithionite.

3.1. *Effect of ATP and ADP*

Figs.1c and 1d and fig.2 traces c and d show the effects of 9 mM ATP or ADP on the rate of reduction

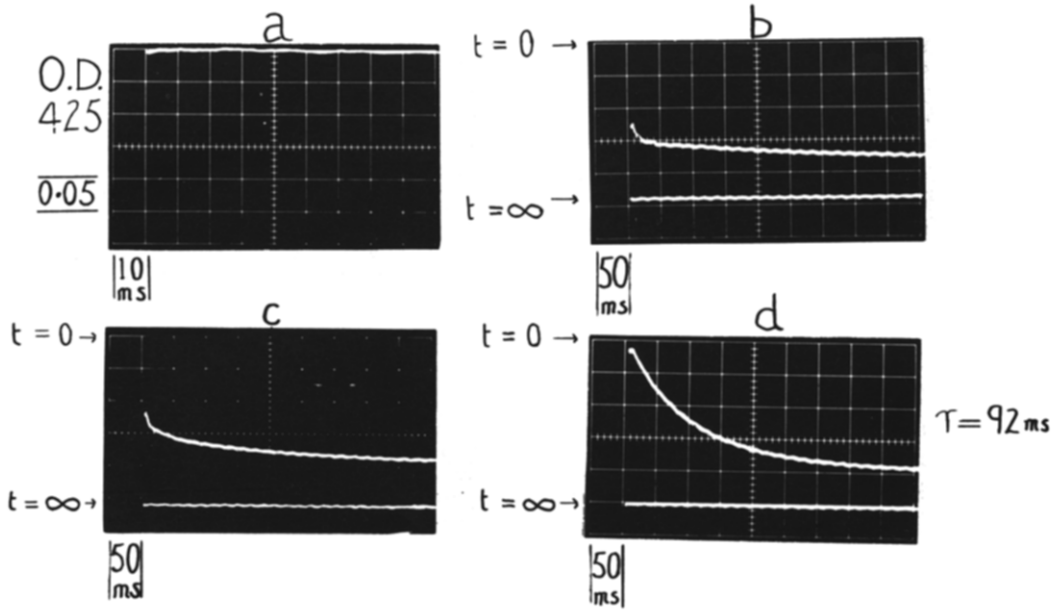

Fig.1. Stopped-flow spectrophotometry: oscillographs of the optical changes associated with the reduction of Ac2-ox by sodium dithionite. In each case one syringe contained Ac2-ox (78 μM) (the concentration of Ac2-ox was calculated using a mol. wt of 64 000 [10]) in 25 mM Tris buffer, pH 7.4, containing 10 mM $MgCl_2$ and 70 μM dithiothreitol. This was mixed in the stopped-flow with and equal volume of (a) Tris buffer; (b) buffer + 20 mM $Na_2S_2O_4$; (c) buffer + $Na_2S_2O_4$ + 18 mM ATP and (d) buffer + $Na_2S_2O_4$ + 18 mM ADP. The reaction was followed at 425 nm at 23°C.

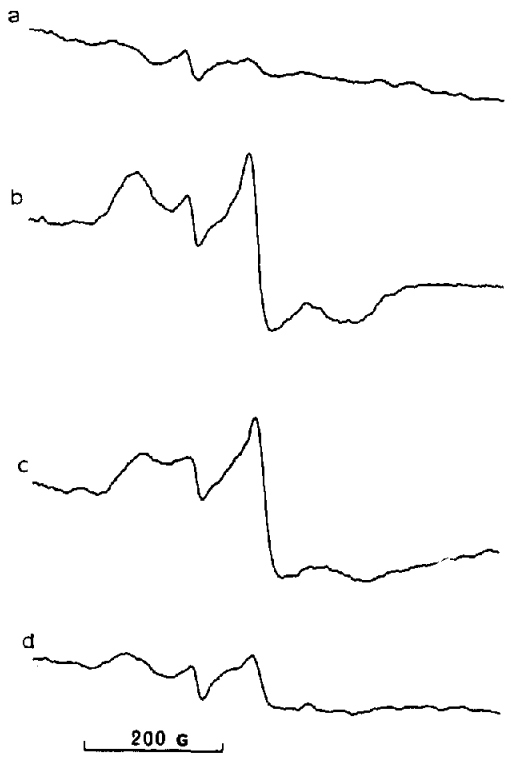

Fig.2. E.p.r. spectra showing the reduction of Ac2-ox by sodium dithionite. The data were obtained by the rapid freezing technique after 20 msec. Reaction conditions and concentrations were as described in fig.1. Spectra were recorded at 9 GHz, 15 K and 15 mW microwave power using a modulating field of 12.5 G.

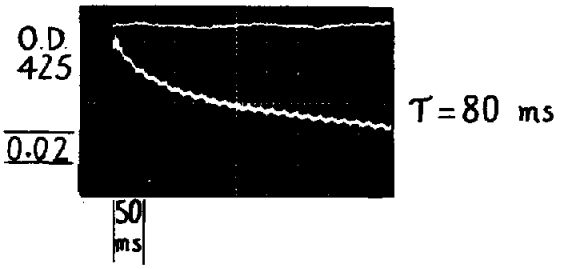

Fig.3. Reduction of oxygen-inactivated Ac2 by sodium dithionite. Ac2-ox (37 μM) in 25 mM Tris buffer, pH 7.4, containing 10 mM $MgCl_2$ and 70 μg/ml of dithiothreitol was inactivated by shaking in air for 10 min. Residual oxygen was removed by flushing with N_2. Upper trace: oxygen-inactivated Ac2 versus Tris buffer. Lower trace: oxygen-inactivated Ac2 versus Tris buffer + 20 mM $Na_2S_2O_4$ (an identical trace was obtained in the presence of 20 mM $Na_2S_2O_4$ + 9 mM ADP).

of Ac2-ox. ADP inhibited both the rapid phase in the stopped flow (τ=92 msec. fig.1d) and the development of the e.p.r. spectrum (fig.2 trace d). Table 1 shows that the extent of development of the e.p.r. spectrum and the rapid phase of reduction were the same in the presence of ADP. ADP did not affect the reduction of oxygen-damaged Ac2: the trace in the presence of 9 mM ADP corresponded exactly to that in fig.3 (τ=80 msec).

It is clear that ATP (fig.1c, fig.2 trace c and table 1) had little or no effect on rate of reduction of Ac2-ox by 10 mM sodium dithionite.

Table 1
Correlation of e.p.r. integration and stopped-flow rapid-phase amplitude measurements in the reactions of Ac2-ox with $Na_2S_2O_4$ described in figs.1 and 2.

Reaction time	Reactant	Relative e.p.r. integration intensities of Ac2 %	Corresponding % change in absorbance at 425 nm (first phase)
30 sec	$Na_2S_2O_4$	103	100
20 msec	$Na_2S_2O_4$	92	100
20 msec	$Na_2S_2O_4$ + ATP	100	100
20 msec	$Na_2S_2O_4$ + ADP	19	20

100% e.p.r. integration intensity corresponds to 0.24 electrons/mol of protein and 100% absorbance change is equivalent to a difference between the extinction coefficients of oxidised and reduced proteins of 2.25 mM^{-1} cm^{-1}.

The e.p.r. signals in the presence of ATP or ADP (fig.2 traces c and d) do not show the full change of symmetry associated with binding of nucleotide to Ac2. This may be due to incomplete reaction but recently observed discrepancies between rapidly frozen (about 1 msec) and manually frozen (several seconds) samples cast doubt on this interpretation. In other experiments, rapidly-frozen samples failed to show the expected conformation change after 5 min reaction of Ac2 with ATP or ADP (or of Ac2-ox with sodium dithionite + ATP or ADP), whereas similar manually frozen samples did so. Similar observations have been made with Kp2 protein (B. E. Smith and D. J. Lowe, unpublished) and, independently, with Cp2 protein, where the effect was attributed to temperature-induced pH changes (W. H. Orme-Johnson and L. Davies, personal communication). Alternatively, such effects could be caused by temperature-induced conformational changes and/or vitrification phenomena dependent upon freezing rate. Temperature induced conformation changes have been demonstrated in the Kp nitrogenase system [11].

4. Discussion

Four important observations are reported in this paper. Firstly, the reduction of Ac2-ox by sodium dithionite occurs in several phases, the first of which is extremely rapid and corresponds to the maximum development of a characteristic e.p.r. signal. Secondly, this phase, like the e.p.r. signal, is not apparent in oxygen-damaged, enzymically-inactive Ac2. Thirdly, the fast phase and the appearance of the e.p.r. signal were inhibited by Mg-ADP but not by Mg-ATP. Finally, there are further slow absorbance changes which do not have a corresponding e.p.r. signal change.

We conclude that the rapid phase of reduction and the characteristic e.p.r. signals are properties of enzymically active protein and that Mg-ADP binds and induces a conformation change in Ac2-ox in less than 1 msec. This conformation, like enzymically inactive protein, is incapable of being reduced rapidly and this could be a site of Mg-ADP control of nitrogenase function, contributing to the relatively slow rate of reaction I in the mechanism of nitrogenase action.

Rapid-freezing e.p.r. spectroscopy on functioning nitrogenase systems shows that the Fe protein is apparently in the oxidised state during enzyme turnover [4]. Since the turnover time is approximately 500 msec per electron, it follows that the rate of apparent reduction of oxidised Fe protein during nitrogenase function is slower than both the rapid phase and the Mg-ADP-inhibited phase with Ac2-ox in the absence of Acl. Presumably, other reactions (e.g. complex formation with Acl [14], deadenylation or dephosphorylation) affect the rate of reduction of Ac2-ox during nitrogenase function.

Since the kinetics of the slow phases remain essentially unaltered upon oxygen inactivation, it is likely that these phases are associated with inactive protein (a detailed kinetic analysis of this complex system shows that SO_2^- is the active reductant: Thorneley, Yates and Lowe, in preparation). We have not been able to establish that the percentage of the fast phase in the overall absorbance change on reduction of Ac2-ox is proportional to enzymic activity. Similarly it is unwise to use the percentage integration of the e.p.r. signal of Ac2 (0.24 electrons per mole) as a criterion of protein activity: the integrated intensity of the e.p.r. spectra of Kp2 protein preparations could not be shown to be proportional to enzymic activity [4]. There may be additional sites which control the enzymic activity of the Fe protein.

This paper provides evidence for the preferential binding of Mg-ADP rather than Mg-ATP to Ac2-ox and this is consistent with the coupling of ATP hydrolysis to electron transfer between the component proteins.

Acknowledgements

We thank Professor J. R. Postgate for reviewing the manuscript and useful discussion, Dr R. C. Bray for the use of e.p.r. facilities and Mrs Frances Campbell for technical assistance.

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