SPECIFIC SPIN-LABELLING OF THE ENERGY-COUPLING SYSTEM OF MITOCHONDRIA

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Received 12 August 1974

1. Introduction

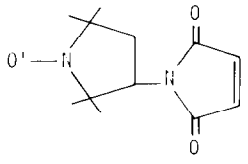
Specifically in the conformational coupling hypothesis of Boyer [1], and implicitly in other hypotheses of oxidative phosphorylation, conformational changes are expected in the coupling factors during electron transport. To observe such changes, it is necessary to use extrinsic probes of the coupling factors because these proteins, unlike the electron-transfer components of the mitochondria, do not themselves contain useful chromophores. Probes that can be attached specifically to coupling factors include aurovertin [2–5], 7-chloro-4-nitrobenzo-2-oxa-1,3, diazole [6] and *N*-(2,2,6,6-tetramethyl-1-oxypiperidyl)-*N'*-(cyclohexyl)-carbodiimide [7]. However, all of these probes have a disruptive effect on the coupling system.

The studies related below describe the attachment of a covalently-bound paramagnetic probe ('spin-label' [8] to mitochondrial ATPase and its natural inhibitor protein [9] without any detectable change in their activities. Such labelled proteins can be used to study interactions with ligands and other proteins, particularly those of the mitochondrial membrane.

2. Materials and methods

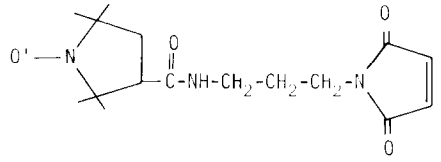
The following probes were used:

PROBE I



3-maleimido-2,2,5,5,-tetramethyl-l-pyrrolidinyloxyl ('short maleimide')

PROBE II



3-[(3-maleimidopropyl)carbamoyl]-2,2,5,5,-tetramethyl-l-pyrrolidinyloxyl ('long maleimide')

Mitochondrial ATPase was prepared by the method of Datta and Penefsky [10] or Knowles and Penefsky [11]. The ATPase inhibitor was prepared and assayed by the method of Horstman and Racker [12]. Combination of ATPase with the inhibitor was carried out as described by Van de Stadt et al. [13]. 'S' and 'SU' particles were prepared as described by Racker and Horstman [14] and the ATPase bound to 'SU' particles by a modification of the method of Bulos and Racker [15]. Submitochondrial particles reconstituted with ATPase and/or inhibitor were suspended in a solution containing 66 mM triethanolamine hydrochloride, 40 mM sucrose, 40 mM KCl, 4 mM $MgCl_2$ and 1.4 mM EDTA at pH 7.4.

The mitochondrial ATPase was labelled as follows. About 300 μg of PROBE I or II (a large excess) was added to 3 ml of a solution of 3 mg/ml ATPase in 0.25 M sucrose, 10 mM Tris–acetate, 4 mM ATP, 2 mM EDTA and 0.2 M KNO_3 at pH 7.5. The probe was dissolved by thorough mixing and the mixture incubated at 30°C for 2 hr. This procedure was based on one described by Dr Sheila Ferguson (personal communication). The protein was separated from free probe and ATP by repeated precipitation with cold saturated ammonium sulphate solution as described by Harris et

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al. [16]. The ATPase activity was assayed as described by Pullman et al. [17]. The probes were not removed from the enzyme by filtration through Sephadex G25 or by prolonged dialysis at room temperature. However, as these processes result in a loss of activity of the ATPase [4,16] and a loss of tightly bound ATP (Rosing, J., Harris, D. A. and Slater, E. C., unpublished), they were not routinely used from separation of free probe from the enzyme.

The ATPase inhibitor was labelled with PROBE I only. The method of labelling was as above except that 3 ml of the mixture contained 300 μg probe, 200 μg/ml inhibitor, 0.25 M sucrose and 10 mM Tris–sulphate at pH 6.0. Free probe was removed by dialysis at 0°C.

Loss of activity of either ATPase or of inhibitor was not observed during any of the labelling procedures. The extent of labelling of the protein was estimated by double integration of the central peak of the ESR signal of a solution of the protein. Total protein sulphydryl groups after dispersal in dodecyl sulphate were measured by the method of Habeeb [18]. Soluble protein was estimated by the method of Lowry et al. [19], and insoluble protein by the biuret procedure of Cleland and Slater [20].

Oligomycin was a gift of the Upjohn Chemical Co. and aurovertin was prepared by Dr R. M. Bertina in our laboratory [21].

3. Results and discussions

The ESR spectrum of the maleimide PROBE II in water is shown in fig. 1. The hyperfine splitting of the lines was 16.5 G.

Fig. 2 shows ATPase labelled with the maleimide probes. When the short maleimide (I) is used (fig. 2A), a two-component spectrum is observed, suggesting that this probe may be bound at two sites of the protein. The longer probe gives a one-component spectrum (fig. 2B(1)), and the maximum labelling obtained after prolonged incubation was 0.9 mole probe solidus mole protein, suggesting that this bulkier probe is bound at only one site. No change in free sulphydryl groups (of which there were 8.5–9.0 per mole F_1) was observed after labelling.

The observed spectrum indicates that the nitroxide group becomes partially immobilized when the probe

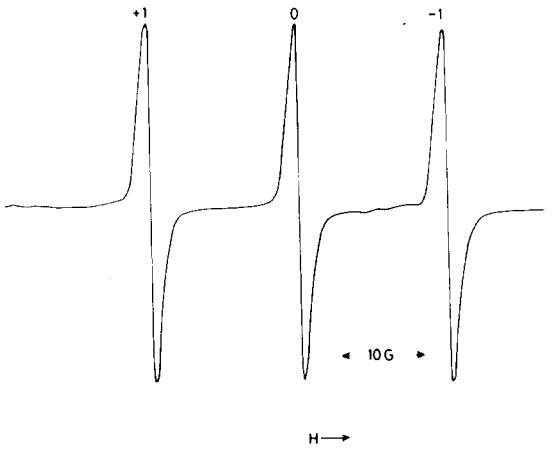

Fig. 1. Spectrum of PROBE II in water. A 10 μM (——) solution of PROBE II (long maleimide probe) in water was placed in a Varian aqueous sample cell No. E248 (gap 0.3 mm) and the spectrum taken at room temperature on a Varian E9 ESR spectrometer. Field 3357 ± 50 G, microwave frequency 9.448 GHz, modulation amplitude 1 G for this and all other spectra.

binds to the protein. The nitroxide group of the probe is in an aqueous environment, as shown by a hyperfine-coupling constant of 16 G for the labelled protein, and the complete reducibility of the probe by ascorbate.

The spectrum is sensitive to gross changes in the protein structure. In 10% sodium dodecyl sulphate, for example (fig. 2B(2)), a change towards higher immobilization is observed, which is surprising. As expected, precipitation of the protein with 50% ammonium sulphate solution leads to immobilization of the probe (fig. 2B(3)).

The ATPase inhibitor labelled with PROBE I gives a fairly mobile one-component spectrum (fig. 3A). Again, the probe occupies an aqueous environment. About 0.7–0.9 mole probe is bound per mole inhibitor with no change in activity. As seen in spectrum 3B, dodecyl sulphate again leads to a clear immobilization of the nitroxide moiety. The inhibitor contains no sulphydryl groups.

Soluble effectors such as adenine nucleotides, Mg^{2+} aurovertin and dinitrophenol did not affect the spectrum of the labelled ATPase.

Labelling of the ATPase did not impair its ability to bind to SU particles. After a short incubation of SU particles with labelled ATPase, both the label and the

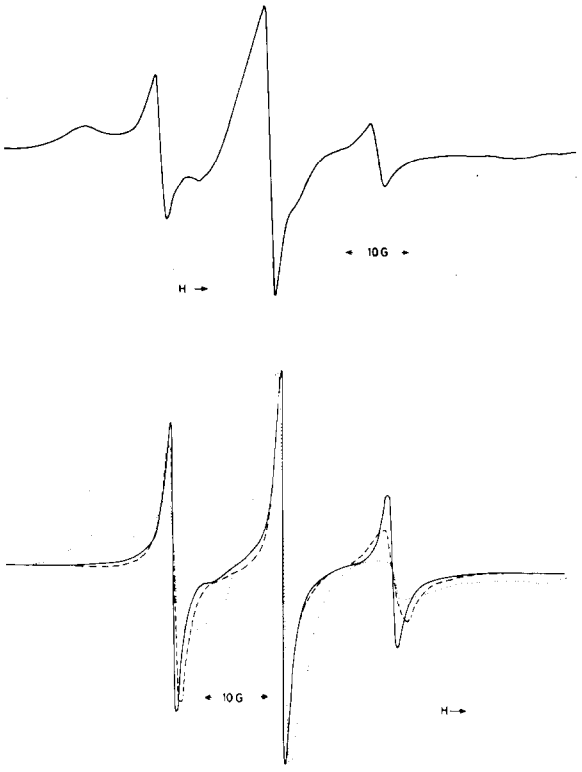

Fig. 2. Spectrum of ATPase labelled with PROBES I and II. ATPase was labelled to 0.6–0.8 mole probe per mole ATPase, and the spectra taken at a concentration of 2 mg protein/ml in 250 mM sucrose, 10 mM Tris acetate, 2 mM EDTA, pH 7.5. A. ATPase labelled with PROBE I; B. (1) (——)ATPase labelled with PROBE II. (2) (- - - -) as (1) + 10% sodium dodecyl sulphate; (3) (. . . . .) as (1) 50% saturated with $(NH_4)_2SO_4$ at pH 7.5.

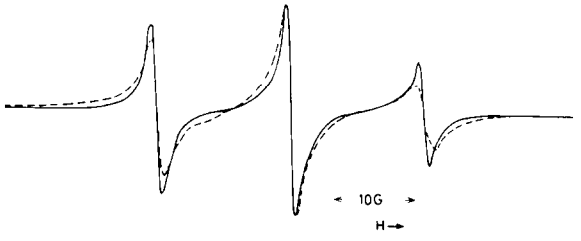

Fig. 3. Spectrum of ATPase inhibitor labelled with PROBE I. The ATPase inhibitor was labelled with PROBE I ('short maleimide') to an extent of 0.7–0.9 mole probe/mole inhibitor, and the spectrum taken at a protein concentration of 150 μg/ml. A. (——) medium as in fig. 2A; B. (- - - - -) medium as in fig. 2B(2).

ATPase activity were found in the particulate fraction (sedimenting at 100 000 *g*). The ATPase activity was inhibited more than 95% by oligomycin, showing that the labelled enzyme assumes its correct orientation with respect to OSCP and CF_0 [22].

Fig. 4 shows the spectrum of labelled free ATPase (fig. 4A) as compared to that of the same preparation bound to particles (fig. 4B). A slight immobilization of the label is seen. The hyperfine splitting is unaltered in the two spectra, suggesting that the probe is not 'trapped' between the ATPase and the membrane proteins in a hydrophobic pocket. It is, therefore, probable that on binding to stripped membranes the ATPase undergoes a conformational change that is sensed by this probe. A further immobilization of the probe was sometimes seen on binding ATPase inhibitor to SU particles reconstituted with labelled ATPase but this was small, and not always visible.

Adenine nucleotides, phosphate, aurovertin, oligomycin, antimycin and succinate (which reduces the particles to anaerobiosis under these conditions) had no effect on the spectrum of the particles, either when tested alone or in combination. The probe was not reduced over the period of the experiment.

Labelling of ATPase inhibitor with PROBE I had no apparent effect on its inhibitory properties or its ability to bind to S particles. The spectra of free and

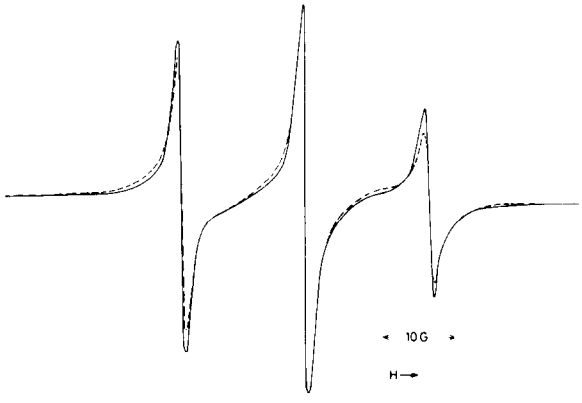

Fig. 4. Spectrum of labelled free and membrane-bound ATPase. A. (——) ATPase labelled with PROBE II as in fig. 2B(1); B) (- - - - -) ATPase labelled with PROBE II and recombined with SU particles as described under Materials and methods. Protein concentration about 20 mg/ml.

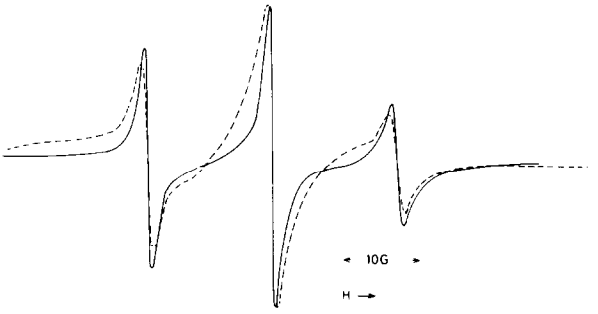

Fig. 5. Spectrum of labelled free and membrane-bound ATPase inhibitor. A. (——) inhibitor labelled with PROBE I as in fig. 3A, or labelled inhibitor recombined with excess free ATPase as described Materials and methods. Protein concentration about 3 mg/ml. B. (- - - - -) labelled inhibitor recombined with S particles as described under Materials and methods. Protein concentration about 20 mg/ml.

bound forms of labelled inhibitor are shown in fig. 5. The spectrum of inhibitor bound to ATPase is identical to that of free inhibitor (fig. 5A), but on binding the inhibitor to particles a clear immobilization of the probe is seen (fig. 5B). This may reflect a difference in the binding site for inhibitor between particle-bound and free ATPase. However, it is difficult to rule out a binding of the inhibitor to additional (non-specific) sites in the particles which may result in this immobilization.

In summary, we can state that mitochondrial ATPase and its naturally occurring inhibitor protein can be labelled with spin-labelled maleimides, each at a single, non-sulphydryl site, without loss of activity. The structure of the ATPase appears to change on binding to stripped particles in such a way that the label attached to it becomes more immobilized and the binding site for inhibitor is altered. The inhibitor also seems to alter in configuration on binding to particles deficient in inhibitor protein (S particles).

An important conclusion to be drawn from these experiments is that, without considerable increase in sensitivity, stoichiometric spin labelling of coupling components is liable to prove too slow and insensitive a method to investigate energization and other state changes in mitochondrial systems, owing to the high concentrations of particles required and the length of time necessary for taking a spectrum. Even in the presence of inhibitors, O_2 or added ATP was used up long before measurement of the spectrum was complete.

Acknowledgements

We wish to thank Mr B. van Swol for technical assistance. This work was supported in part by grants from the Netherlands Organization for the Advancement of Pure Research (Z.W.O) under the auspices of the Netherlands Foundation for Chemical Research (S.O.N.), and the NATO research grant No. 589. D.A.H. is the recipient of a CIBA-Geigy European Fellowship.

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